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14. ABSTRACT Following spinal cord injury there is a protracted accumulation of iron at the site of the injury. Iron plays a role in multiple damaging pathways following injury but is also essential for maximal oligodendrogenesis. The current experiments investigate the therapeutic potential of iron chelation following traumatic spinal cord injury. Our initial research indicates that if iron chelation with the Federal Drug Administration (FDA) approved drug Exjade is begun approximately 1.5 hours after a spinal cord injury there is limited improvement in locomotor function and increased spared of grey matter. Since iron accumulation occurs in a protracted manner, we also investigated the potential of delayed the start of Exjade treatment by 1 week. This paradigm, however, did not result in any of the improvements observed in the early onset treatment. Increasing the dose of Exjade to 320mg/kg/day resulted in increased mortality and therefore was discontinued. In attempts to improve the effects of iron chelation, we have conducted experiments using a different FDA approved chelator, deferiprone. Following either contusive or hemisection spinal cord injury, deferiprone improves functional and histological outcome.			
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Introduction:

It is widely recognized that intraparenchymal bleeding occurs after traumatic spinal cord injury (SCI) (Noble and Wrathall 1989). This, along with cellular debris released as cells die, increases intraspinal iron concentration. While iron is an essential element required for most basic cellular functions, iron is also highly reactive and quite toxic when present in supra-physiological levels. This application seeks to dissect the deleterious aspects of iron elevation from those needed for glial replacement after mid-thoracic contusion injury or cervical lateral hemi-section injury. Our pilot data reveal that iron levels do not actually rise immediately after SCI but in a delayed fashion. Therefore we will test the hypothesis that delayed administration of an FDA approved iron chelator will preserve the early glial genesis response by allowing sufficient iron to be present for proliferative processes, and that the delayed treatment will effectively dampen the more chronic deleterious aspects of excess iron. We will perform detailed anatomical and behavioral studies to determine if iron chelation provides improvement in any relevant outcome measures following spinal contusion or hemi-section injury.

BODY

Aim 1: Using a rat spinal contusion model, compare the efficacy of immediate (1.5hrs) versus delayed (1 week) iron chelation on anatomical and functional outcome. (Months 1-18)

Task 1: Exp 1.1) Evaluate the effect of iron chelation on acute anatomical outcome (months 1-6)

- n=8 rats/group x 6 experimental groups = 48 rats (spinal cords will be used for Tasks 1b-1d)
 - Week 1 Group: n=8 each receive iron chelator, vehicle, nothing on days 0-6 post-injury
 - Week 2 Group: n=8 each receive iron chelator, vehicle, nothing on days 7-14 post-injury

1a: Perform thoracic level 8 (T8) spinal contusion injuries and drug/vehicle treatments (month 1)

We completed the injuries and drug/vehicle treatments as stated. The open field locomotor data collected during this time is presented below. The Basso Beattie Bresnahan (BBB) open field locomotor rating scale (Basso, Beattie et al. 1995) was used to test the effect of Exjade treatment on locomotor recovery. Analysis of the early treatment, which was begun 1.5 hours post-injury, revealed that the 160mg/kg/day dose of Exjade improved locomotor function compared to control (Figure 1A). Delayed treatment with Exjade, which was begun 7 days post-injury, did not improve open field locomotion (Figure 1B). Intriguingly, rats receiving vehicle (grape juice) performed significantly worse than those receiving no treatment suggesting that the vehicle may have confounded results (Figure 1B).

Figure 1: Analysis of locomotor function following spinal cord injury in animals given the iron chelator Exjade, vehicle (grape juice) or nothing (control).

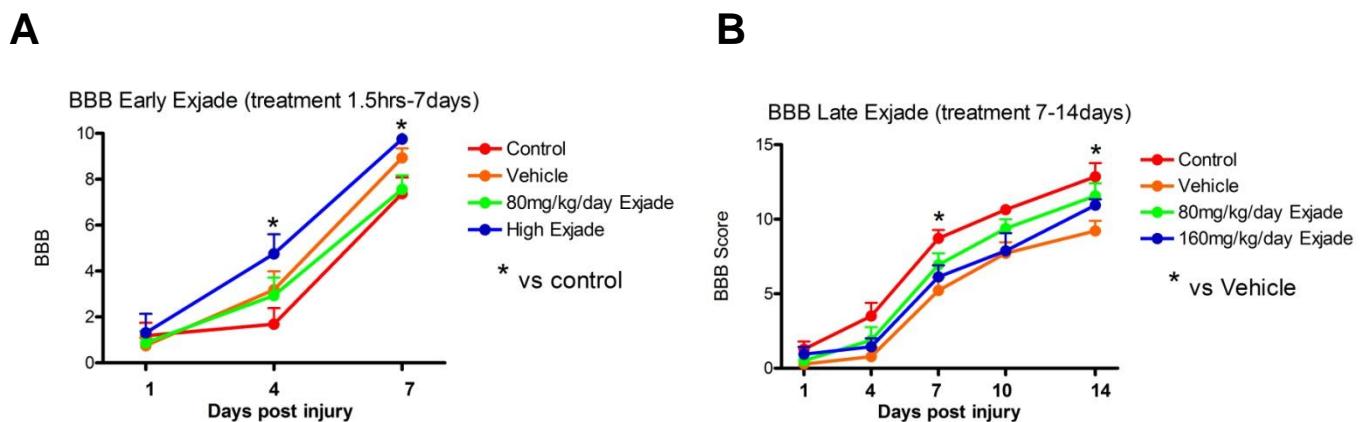


Figure 1: Analysis of locomotor function following spinal cord injury in animals given the iron chelator Exjade. A) Animals receiving Exjade at 160mg/kg/day beginning 1.5 hours after the injury had significantly better locomotor performance. B) Delaying the initiation of Exjade treatment by 1 week resulted in no improvements in locomotor performance. Interestingly, animals which were given the grape juice vehicle beginning 1 week after the injury performed significantly worse than controls. *= $p<0.05$

1b: Perfuse animals, collect spinal cords, prepare spinal cords for immunohistochemistry, cut frozen sections

(month 2)

We completed the perfusions, collection of spinal cord, and preparation of tissue for immunohistochemistry.

1c: Immunohistochemistry for white matter sparing, progenitor cell proliferation, oligodendrocyte genesis, neurons (grey matter), apoptosis, iron (months 3)

We completed the immunohistochemistry for white and grey matter tissue sparing, macrophage induced inflammation, oligodendrocyte genesis, iron accumulation, and neuron sparing. All markers have been analyzed except for caspase 3 (the apoptotic marker). We had problems labeling for activated caspase 3.

1d: Analyze tissue using light and confocal microscopy (months 4-6)

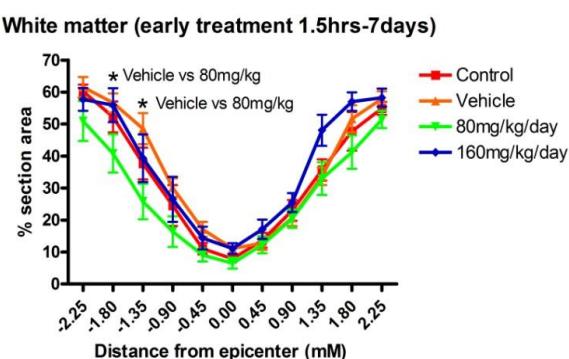
Quantification of these outcomes is presented below in separate sections below for each endpoint.

White matter sparing:

In rats given Exjade beginning 1.5 hours post-injury (early treatment), the lower dose of Exjade (80mg/kg/day) resulted in significantly less white matter sparing in sections rostral to the epicenter compared to vehicle treated rats (Figure 2A). The reason for this is currently unknown. The higher dose of Exjade (160mg/kg/day) had no significant effect on white matter sparing (Figure 2A). In rats given Exjade beginning 1 week post-injury, the higher dose Exjade group (160mg/kg/day) had significantly less white matter at 2.25mm rostral and caudal to the epicenter (Figure 2B). It is possible that delaying treatment by 7 days did not allow sufficient protection from the earlier accumulation of iron.

Figure 2: Analysis of white matter tissue sparing following spinal cord injury in animals administered the iron chelator Exjade

A



B

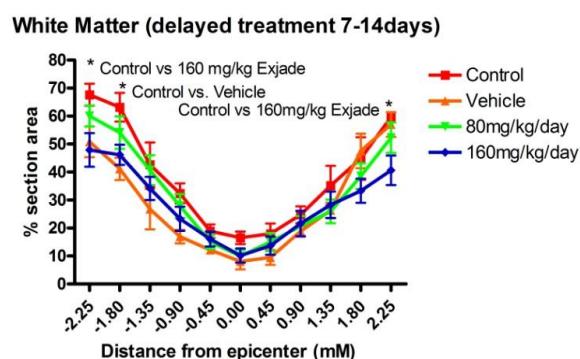


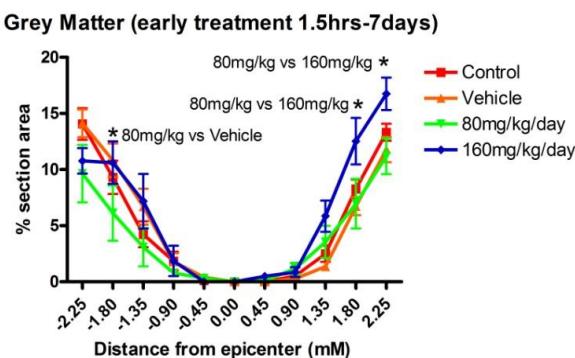
Figure 2: Analysis of white matter tissue sparing following spinal cord injury in animals administered the iron chelator Exjade. Analysis of white matter tissue sparing was performed across the entire rostral to caudal extent of the lesion in both the early treatment (1.5hrs-7days) and delayed treatment (7-14days) groups. **A)** Statistical analysis revealed that following the early treatment with Exjade at 80mg/kg/day resulted in less white matter rostral to the injury as compared to the vehicle treated group. **B)** Analysis of the delayed treatment groups revealed that animals receiving Exjade at 160mg/kg/day had less white matter sparing rostral and caudal to the injury compared to control animals which received no intervention. In addition, animals that received the grape juice vehicle also had less white matter sparing rostral to the injury compared to control animals. * $p<0.05$

Grey matter sparing:

In rats treated with Exjade beginning 1.5 hours post-injury (early treatment), the lower dose (80mg/kg/day) caused a significant reduction in gray matter sparing rostral to the epicenter, similar to reduced white matter sparing shown above (Figure 3A). Interestingly, caudal to the epicenter the 160mg/kg/day dose resulted in significantly increased gray matter sparing compared to the 80mg/kg/day dose (Figure 3A); this result was not significantly different from controls, however. No significant differences in gray matter sparing were noted in the delayed treatment group with the exception of lower gray matter rostral to epicenter in the vehicle group. (Figure 3B)

Figure 3: Analysis of grey matter tissue sparing following spinal cord injury in animals administered the iron chelator Exjade

A



B

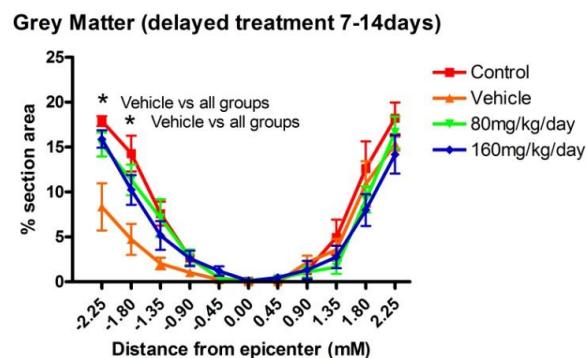


Figure 3: Analysis of grey matter sparing following spinal cord injury in animals given the iron chelator Exjade. Grey matter sparing was quantified across the rostral to caudal extent of the lesion in both the early treatment (1.5hrs-7days) and delayed treatment (7-14days) groups. **A)** Analysis of the early treatment groups revealed that animals receiving the 80mg/kg/day dose of Exjade had significantly less grey matter sparing rostral to the epicenter compared to vehicle treated animals. Additionally, animals in the early treatment groups which were given Exjade at 160mg/kg/day had significantly more grey matter sparing caudal to the epicenter compared to animals which received Exjade at 80mg/kg/day. **B)** Analysis of the delayed treatment groups revealed that animals in the vehicle treatment group had significantly less grey matter rostral to the injury compared to the control treated animals. * $p<0.05$

Oligodendrocyte Numbers:

We quantified the number of oligodendrocytes rostral to the epicenter, since our previous work has shown oligodendrocyte genesis occurs after SCI (McTigue, Wei et al. 2001; Tripathi and McTigue 2007). Cell counts were performed along white matter lesion border, gray matter lesion border, and spared white matter. The results revealed no significant increase in either Exjade treated groups. While these results suggest that oligodendrocyte genesis was not enhanced by Exjade, this is an important finding since there is the possibility that iron chelation

could reduce oligodendrocyte genesis after injury. Thus, it appears that treatment with Exjade did not reduce oligodendrocyte generation.

Figure 4: Analysis of oligodendrocyte genesis following spinal cord injury in animals administered the iron chelator Exjade

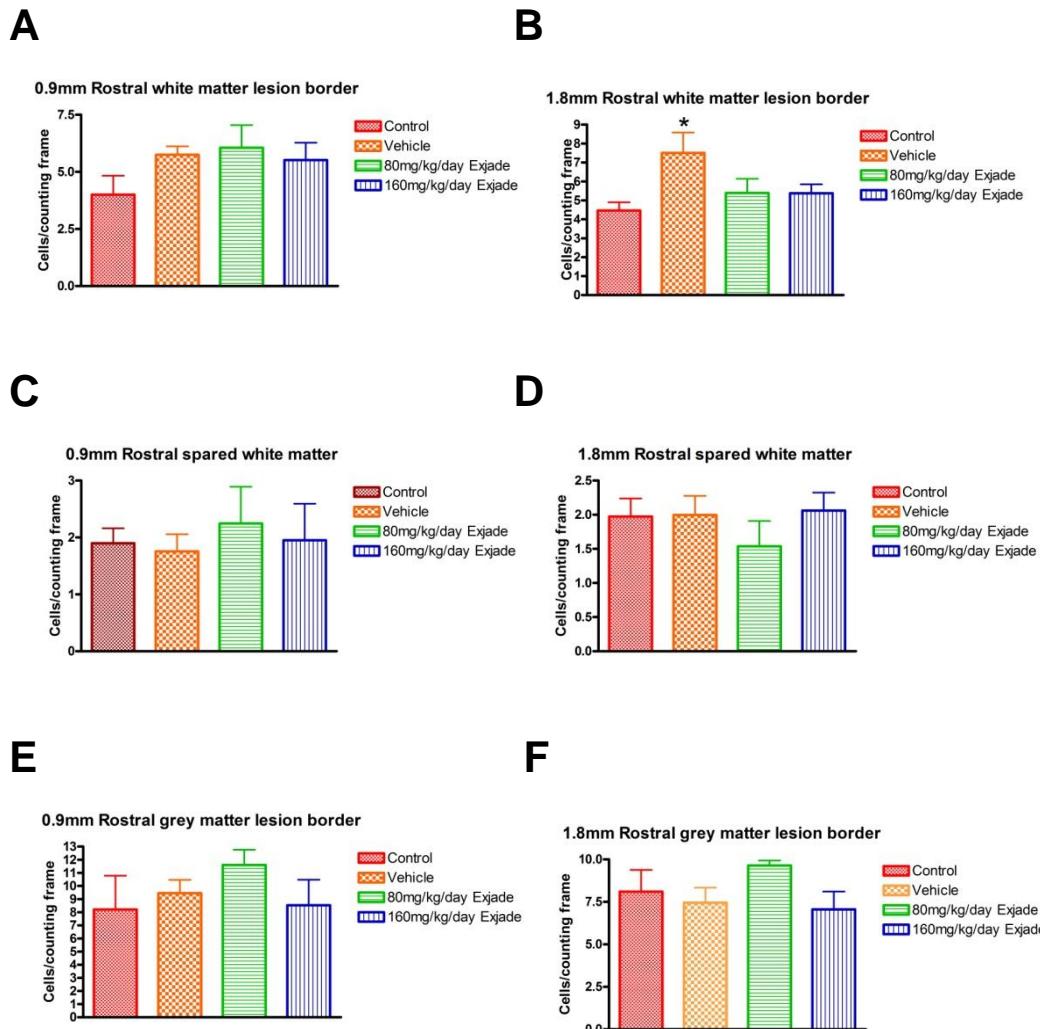


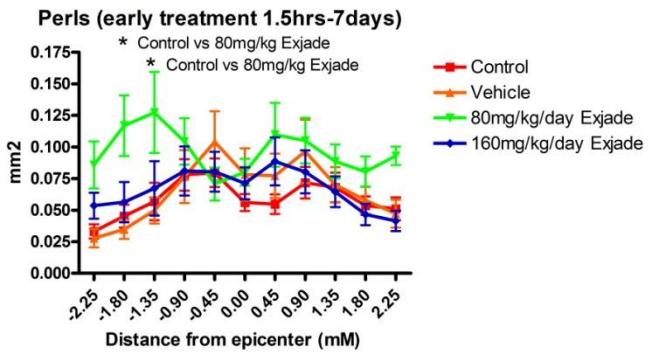
Figure 4: Analysis of oligodendrocyte genesis following spinal cord injury in animals given the iron chelator Exjade. Oligodendrocytes were quantified rostral to the epicenter (0.9mm & 1.8mm) in animals that received early treatment beginning 1.5 hours after the injury. Cells were counted in the white matter lesion borders (A,B), spared white matter (C,D) and grey matter lesion borders (E,F). Analysis of the white matter lesion border at 1.8mm rostral to the epicenter revealed significantly more oligodendrocytes in the vehicle treated group compared to the control treated group. No other significant differences were noted. * $=p<0.05$

Iron accumulation:

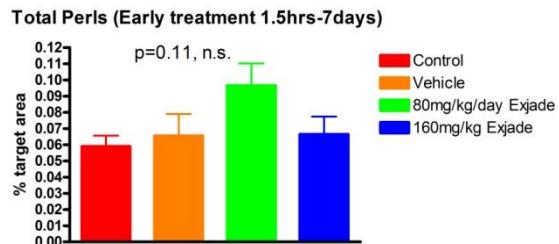
As expected, iron accumulated at the site of the injury and appeared to be predominantly present within macrophages in the lesion. Quantification iron levels using the Perls' stain revealed increased intraspinal iron in the 80mg/kg/day Exjade group rostral to the injury epicenter (Figure 5A); however, this apparent increase was the result of 2/8 rats in this group. Furthermore, analysis of total iron content across the entire rostral to caudal extent of the lesion revealed no significant difference in iron content among the groups (Figure 5B). No differences were observed following the delayed treatment paradigm (Figure 5C).

Figure 5: Analysis of iron accumulation following spinal cord injury in animals administered the iron chelator Exjade

A



B



C

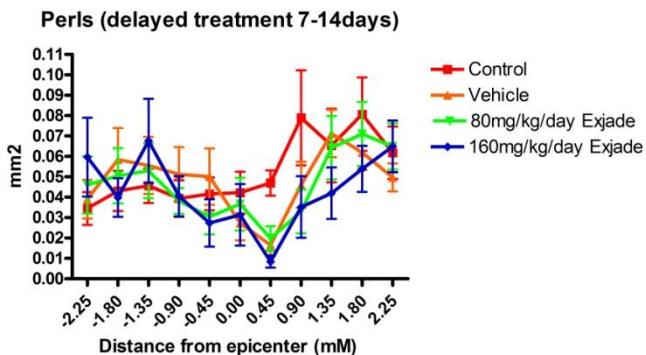


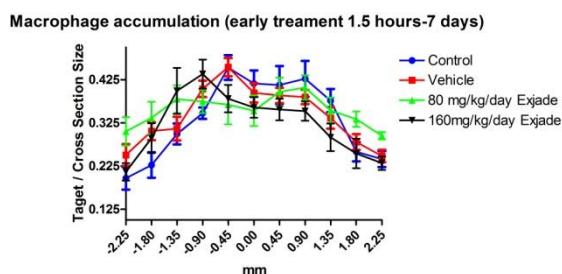
Figure 5: Analysis of iron accumulation following spinal cord injury in animals given the iron chelator Exjade. Perls' staining was performed across the rostral to caudal extent of the lesion to determine the amount of iron present in the spinal cord after injury. **A)** Analysis of the early treatment groups which received Exjade beginning 1.5h post-injury revealed that animals in the 80mg/kg/day Exjade group had significantly more iron rostral to the injury compared to control animals, however, there was no difference in the total area immunoreactive for iron in the spinal cord **(B)**. **C)** No differences were observed between groups in the delayed treatment study, * $=p<0.05$

Macrophage Accumulation:

Following spinal cord injury, macrophages infiltrate the lesion site and accumulate in the spinal cord. Since most iron in the spinal cord appears to be stored by macrophages, the effect of Exjade on macrophage accumulation was analyzed in animals that received Exjade being either 1.5h or 7 days following the injury. No differences were observed between any of the groups in the amount of area immunoreactive for the macrophage marker OX42 (Figure 6).

Figure 6: Analysis of macrophage accumulation following spinal cord injury in animals administered the iron chelator Exjade

A



B

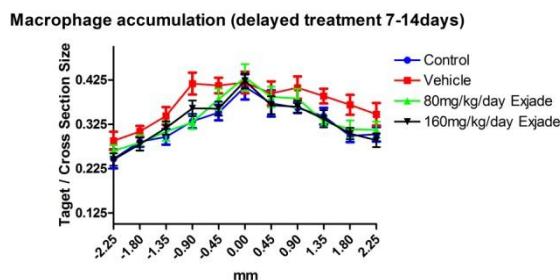


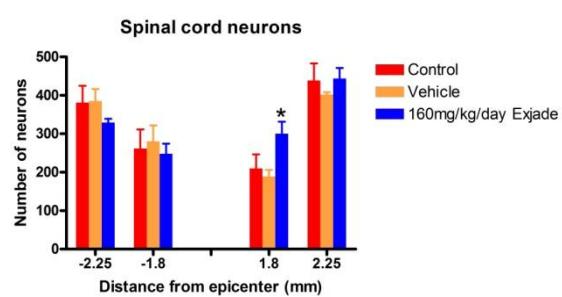
Figure 6: Analysis of macrophage accumulation following spinal cord injury in animals given the iron chelator Exjade. OX42 immunohistochemistry was performed across the rostral to caudal extent of the lesion to determine the accumulation of macrophages in the spinal cord after injury. **A,B)** No differences were observed between any groups in either the early or delayed treatment paradigms.

Neuron counts:

As indicated above in the analysis of grey matter tissue sparing, there was significantly more grey matter sparing rostral to the epicenter in animals given 160mg/kg/day Exjade beginning 1.5h after injury. To assess whether the general increase in grey matter included protection of individual neurons, the neuronal marker NeuN was used to quantify neuron sparing in these animals. Quantification of the total number of neurons revealed significantly more neurons present at 1.8mm caudal to the epicenter in Exjade-treated animals (Figure 7).

Figure 7: Analysis of neuron sparing following spinal cord injury in animals given the iron chelator Exjade

A



B



Figure 7: Analysis of neuron sparing following spinal cord injury in animals given the iron chelator Exjade. NeuN immunohistochemistry was performed across the rostral to caudal extent of the lesion to determine neuron sparing in the spinal cord after injury. **A)** Exjade treatment at 160mg/kg/day beginning 1.5h after injury increased the number of neurons at 1.8mm rostral to the epicenter. **B)** Representative images of NeuN-labeled cross-sections from a rat treated with vehicle or 160mg/kg/day Exjade. * $p<0.05$

Task 2: Exp 1.2) Determine the long-term effect of iron chelation during week 1 or week 2 on functional outcome, tissue sparing and myelination (months 7-18)

- n=16 rats/group x 5 experimental groups = 80 rats (spinal cords will be used for Tasks 2b-2e)
 - Week 1 Group: n=8 each receive iron chelator, vehicle, nothing on days 0-6 post-injury
 - Week 2 Group: n=8 each receive iron chelator, vehicle, nothing on days 7-14 post-injury

2a: Perform T8 spinal contusion injuries and drug/vehicle treatments (months 7-8)

In the previous study, we became aware after that fact that our vehicle (grape juice) may have had unintentionally affected the outcomes. Therefore, prior to initiating long-term studies, we conducted pilot experiments using water as the vehicle for Exjade. In these studies, animals treated with 160mg/kg/day Exjade exhibited improved locomotor recovery during the drug treatment and had significantly better stepping at 7dpi (Figure 8). Thus, studies for Task 2 will be initiated using the new vehicle. We also tested a higher dose of Exjade (320 mg/kg/day), which led to toxicity in 3/6 rats. Thus, future studies used 160mg/kg.

Figure 8: Analysis of post-injury locomotor recovery in animals given the iron chelator Exjade with the use of water as the vehicle

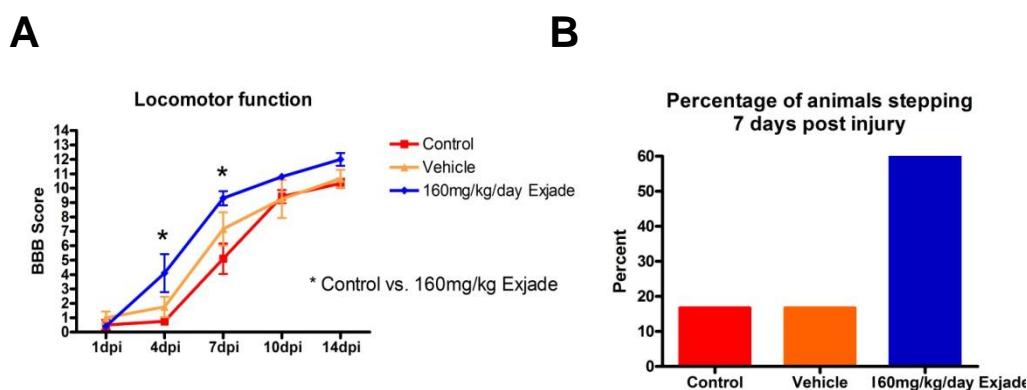


Figure 8: Analysis of locomotor recovery using the BBB scale following spinal cord injury in animals given the iron chelator Exjade with water as the vehicle. Given the finding in our initial studies suggesting complications arising from using grape juice as the vehicle for Exjade, these studies were performed using water as the vehicle. **A)** Analysis of locomotor function revealed no differences between the control and vehicle treated animals. Locomotor recovery was significantly improved rats given Exjade at 160mg/kg/day. **B)** Significantly more Exjade-treated rats could plantar step at 7d compared to both control groups. *= $p<0.05$

Figure 9: Comparison of 1 week versus 2 week Exjade treatment.

Administration of Exjade for 1 week at 160mg/kg/day results in modest improvement in acute locomotor recovery (Figure 8). To determine if improved recovery could be sustained by prolonging treatment, a new cohort of animals was used to compare 1 week versus 2 week treatment with Exjade. However, contrary to our previous data, Exjade treatment did not improve locomotor function in the one week group; there was no detectable change in locomotor performance in rats treated for 2 weeks (Figure 9).

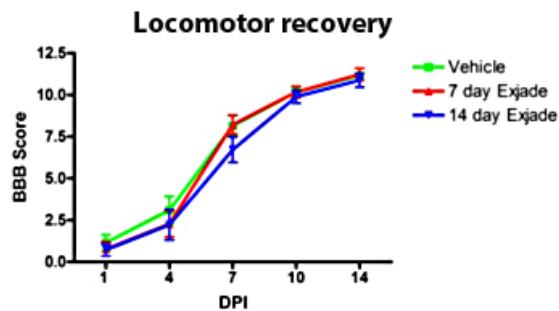


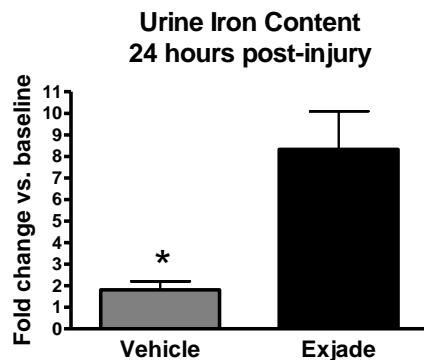
Figure 9: Comparison of 1 week versus 2 week administration of Exjade.

Exjade was given at 160mg/kg/day for either 1 or 2 weeks post-injury injury. Neither treatment group showed any difference in hindlimb function following Exjade administration compared to vehicle controls.

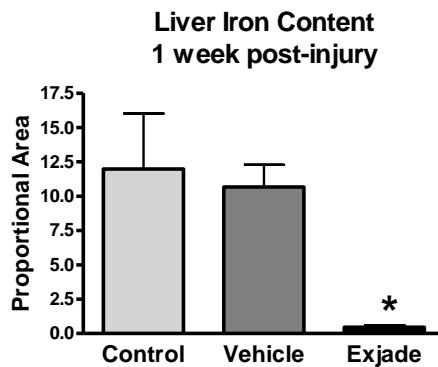
Analysis of liver and urine iron content confirm Exjade is functional after oral administration.

Oral treatment with Exjade should induce peripheral iron chelation. To confirm that the orally delivered Exjade was functional, both urine and liver iron content was determined. Analysis of urine iron content revealed at 24 hours after the first Exjade treatment, iron was significantly increased in the urine. After one week of Exjade treatment at 160mg/kg/day, there was a significant decrease in liver iron content. However, contrary to the effects in the liver, there was no change in intraspinal iron content in Exjade-treated rats. This may be due to an inability of Exjade-bound iron to be cleared from the spinal cord.

A



B



C

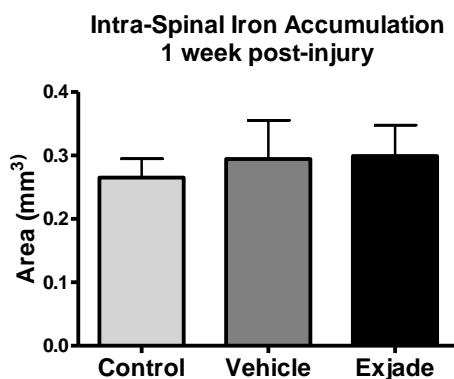


Figure 10: Analysis of liver and urine iron content to confirm Exjade is functional after oral administration. **A)** Urine iron analysis shows that 24 hours after Exjade treatment (160mg/kg/day), iron levels were significantly increased in the urine compared to vehicle-treated rats. **B)** Liver iron content was significantly reduced after 1 week of Exjade treatment at 160mg/kg/day. **C)** Intraspinal iron concentration was not different between Exjade and control groups.

2b: Functional analyses: BBB, activity box, von Frey hair (months 7-9)

Treatment with Exjade at 160mg/kg/day for 1 week did not replicate locomotor improvements after traumatic SCI.

Replication of previously effective dose of Exjade, 160mg/kg/day resulted in no beneficial effect after spinal cord injury. Analysis of hind limb locomotor function with the BBB rating scale showed no benefit of Exjade treatment for 1 week post-injury. Analysis of general locomotor function with automated activity boxes also showed no effect of Exjade treatment. Analysis of hind limb sensory function with Von Frey hairs showed no change in sensory recovery compared to control animals.

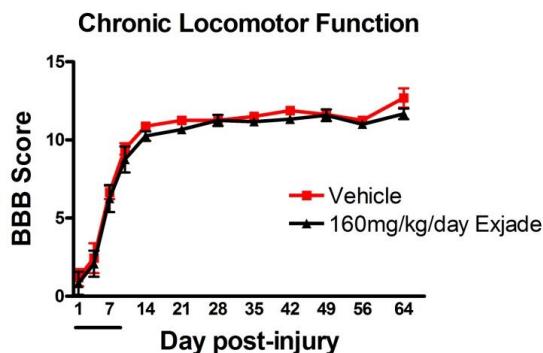
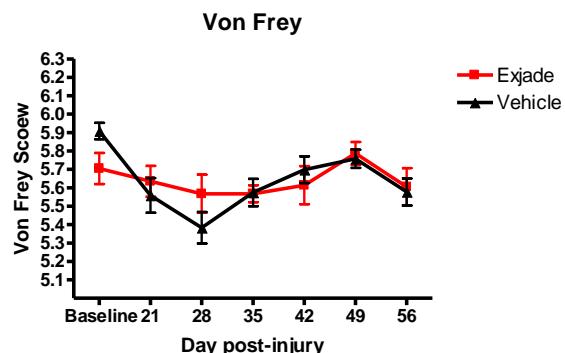
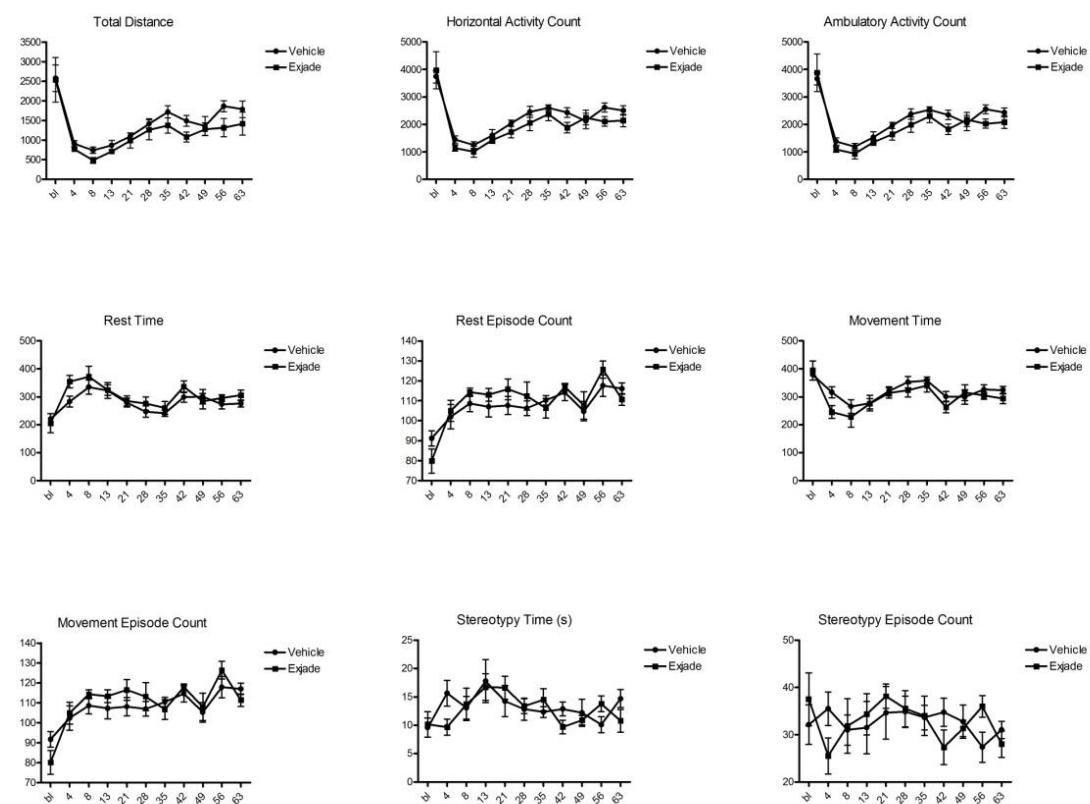
A**B****C**

Figure 11: Treatment with Exjade at 160mg/kg/day for 1 week post-injury did not replicate locomotor improvements after traumatic SCI. A) Treatment with Exjade did not improve hind limb locomotor recovery as assessed with the BBB locomotor rating scale. B) Treatment with Exjade did not alter hind limb sensitivity to Von Frey hair stimulation. C) Treatment with Exjade did not result in any differences in locomotor activity in automated activity boxes.

2c: Perfuse animals, collect spinal cords, prepare half of the spinal cords for immunohistochemistry and cutting frozen sections; prepare other half of spinal cords for epon embedding and cutting semi-thin sections (months 9-11)

We completed the perfusions, collection of spinal cord, and preparation of tissue for immunohistochemistry.

2d: Immunohistochemistry for white matter sparing, progenitor cell proliferation and survival, oligodendrocyte numbers (months 12-13)

Treatment with Exjade did not replicate increased tissue sparing following spinal cord injury.

Replication of the previously effective dose, 160mg/kg/day for 1 week, did not improve grey or white matter sparing after spinal cord injury. Analysis of the amount of spared white matter with the use of the eriochrome cyanine stain showed no improvement following Exjade treatment. Similarly, analysis of spared grey matter with staining for neurofilament showed no differences in grey matter sparing.

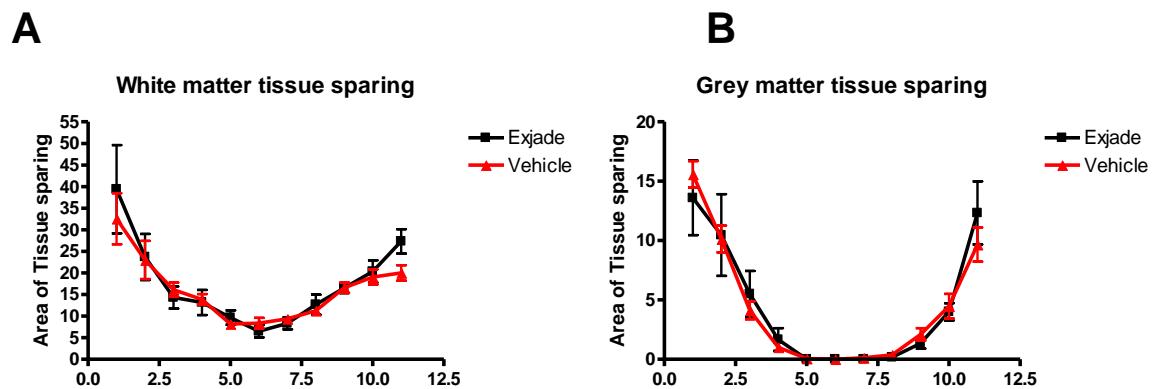


Figure 12: Treatment with Exjade fails to replicate tissue sparing following spinal cord injury.
Analysis of spared white matter tissue (A) and grey matter shows no difference between vehicle and Exjade treated animals.

Our previous experiments using Exjade showed only limited functional improvement (Figures 1 and 8), tissue sparing (Figure 3), and protection of spinal cord neurons (Figure 7). Furthermore, attempts to replicate the protective effects of Exjade and investigate long term changes failed to produce any effect (Figures 9, 11, and 12). We confirmed that the orally administered Exjade chelated iron systemically by increasing urinary iron excretion and decreasing liver iron content (Figure 10). However, contrary to the systemic effects, Exjade did not produce a detectable decrease in intra-spinal iron content (Figure 10). Given this and the variable functional/histological results, we conducted experiments with the FDA approved iron chelator Deferiprone, which has greater membrane permeability than Exjade, which was hoped to provide better chelation of intra-spinal iron since the drug will reach the CNS at higher levels.

Administration of the iron chelator deferiprone leads improvements locomotor function and tissue sparing.

The iron chelator deferiprone was administered intrathecally to provide direct drug administration to the spinal cord while avoiding systemic side effects. Immediately prior to the spinal cord injury a catheter connected to an Alzet osmotic mini-pump was inserted under the dura adjacent to the injury site. Deferiprone was administered continuously throughout the study at a dose of 500ug/kg/day. By 4 days post-injury animals receiving deferiprone showed significant improvements in hindlimb locomotor function. These same animals also showed improvements in white and grey matter tissue sparing and neuron sparing post-injury.

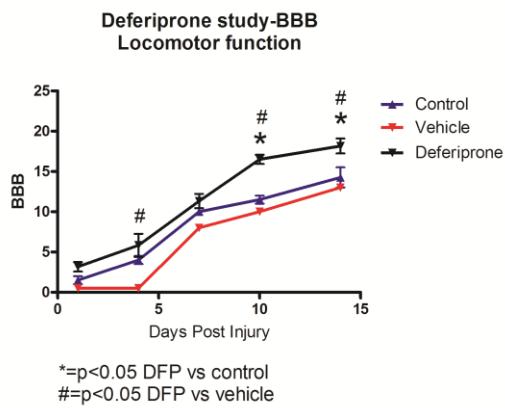
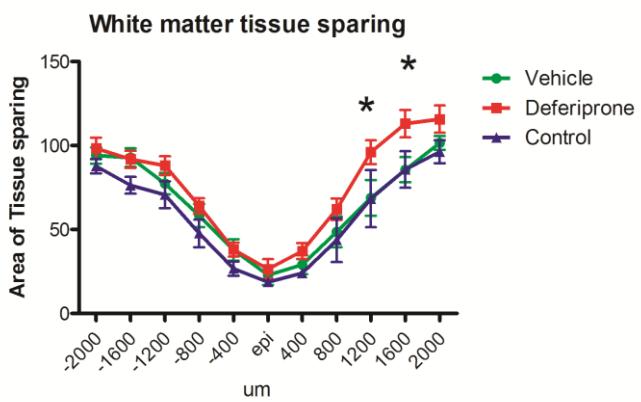
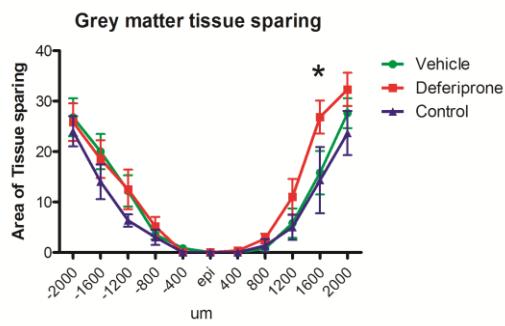
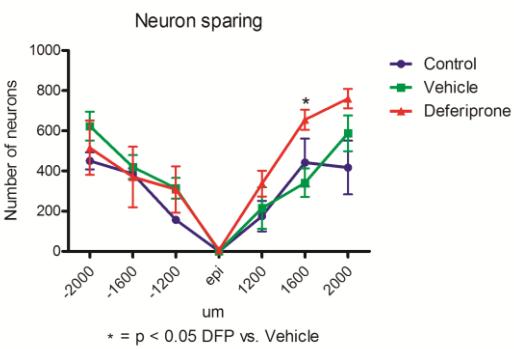
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Figure 13: Treatment with deferiprone results in locomotor improvement and tissue sparing.

Animals administered deferiprone showed improvements in locomotor function by 4 days post-injury (A). Analysis of tissue sparing showed increased white matter (B) and grey matter (C) tissue sparing at 2 weeks post-injury. Quantification of neurons showed that the same regions exhibited spared grey matter exhibited spared neurons.

Administration of the iron chelator deferiprone does not reduce intraspinal iron or ferritin levels.

Following administration of deferiprone at 500ug/kg/day for 2 weeks, injured spinal cord tissue was analyzed for levels of iron and ferritin. Perls staining for iron did not show a significant decrease in iron levels. Furthermore, staining for the iron storage protein ferritin did not show a decrease after deferiprone administration. The lack of a reduction in iron and ferritin levels is similar to what was observed following administration of Exjade, suggesting removing iron from the injured spinal cord may be difficult to achieve. However, the locomotor and histological effects show that administration of an iron chelator can still be effective.

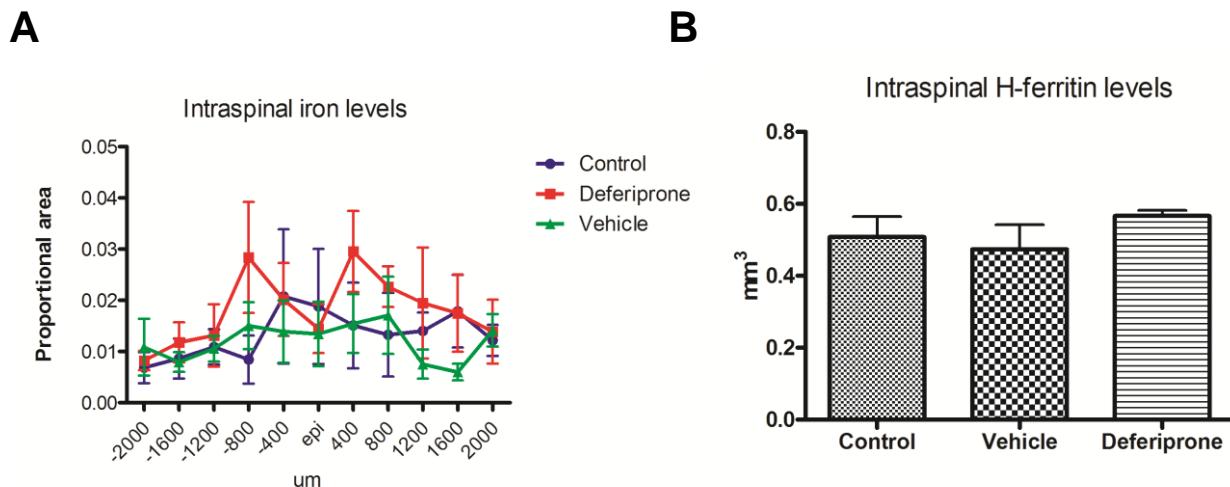


Figure 14: Treatment with deferiprone does not reduce intraspinal iron or ferritin levels.

Animals administered deferiprone did not show a decrease in intraspinal iron (A) or ferritin (B) levels.

Aim 2: Using an axon cut-injury model (spinal hemisection), compare the efficacy of immediate versus delayed iron chelation on anatomical and functional outcome. (months 19-36)

We built upon our preliminary experiments to confirm the validity of the cervical spinal cord hemisection injury model. With this model we are able to create a unilateral injury where right side of the spinal cord is completely destroyed. Over the first week post-injury the injury does not spread to the left side of the spinal cord (Figure 15). Since the injury does not spread contralaterally, we were able to use the left forepaw as a within animal control for our behavioral assessments following iron chelator administration. With this injury model, iron is distributed mainly to the injured side of the spinal cord, and spread both rostral and caudal in areas where tissue sparing was more prevalent (Figure 16).



Figure 15: Representative images of the epicenter following cervical spinal cord hemisection.

Tissue sections are stained with eriochrome cyanine for white matter and neurofilament for grey matter.

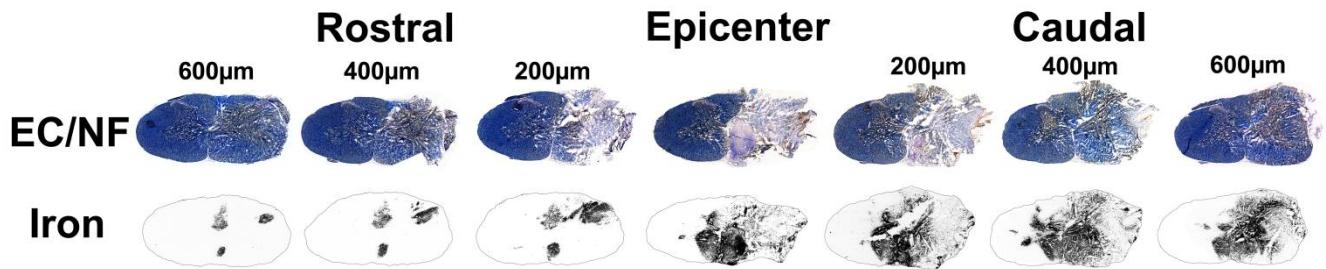


Figure 16: Representative images of the injured tissue at 5 days post-injury. Tissue sections are stained with eriochrome cyanine for white matter and neurofilament for grey matter. Adjacent tissue sections were stained for iron using the Perls technique.

Given our previously mentioned problems with the iron chelator Exjade, the iron chelator deferiprone was used after a unilateral spinal hemisection. Given that this model results in damage the dura, deferiprone was administered using gel foam soaked in the drug. This method allowed for the localized delivery of the drug directly to injured spinal cord. The gel foam was cut into cubes so that surface area of each side was $29.8 \pm 5.5 \text{ mm}^2$. Gel foam was soaked in either 74mM deferiprone or the PBS vehicle for at least 24hours at 37°C to allow complete absorption of the solution. Following injury to the spinal cord, bleeding was allowed to stop and the gel foam was placed directly on the spinal cord with an average time to treatment of 3.6 ± 0.7 minutes. As a control for the administration of gel foam, Control animals were generated that received the spinal cord injury followed by no gel foam treatment.

As expected, the injury to the spinal cord resulted in acute functional impairment in ipsilateral forepaw. To quantify the severity of dysfunction and detect any recovery the Pasta test and Cylinder test were utilized since they directly assess forepaw function. Briefly, the Pasta test looks at the ability of the animal to manipulate a piece of uncooked elbow pasta and this test is able to detect Normal, Abnormal, and Transitional forepaw movement. The transitional movements characterize the earliest signs of recovery. 14 days post-injury there were no differences in Normal forepaw movements (Figure 17A). However, animals that received deferiprone exhibited a greater percentage of transitional movements while eating the piece of pasta (Figure 15B), indicating improved recovery. In contrast, no Control animals and only one Vehicle treated animal exhibited any transitional forepaw movements (Figure 17B). Given the increased percentage of transitional movements, there was a corresponding decrease in Abnormal movements (Figure 17C). Even with the positive effects observed with the Pasta test, no animals from any group exhibited normal plantar forepaw placements during the Cylinder test (Figure 17D), supporting our finding that Normal movements had not returned (Figure 17A). These data suggest that deferiprone treatment is allowing the animals to develop new functional strategies to achieve successful forepaw use.

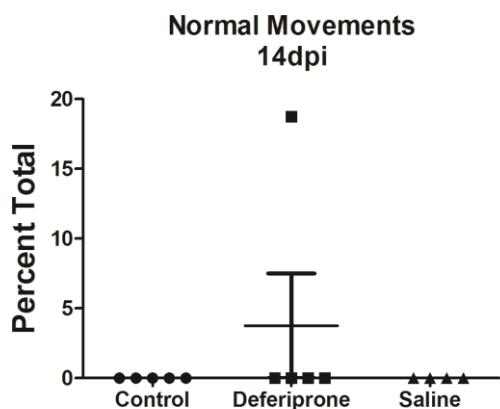
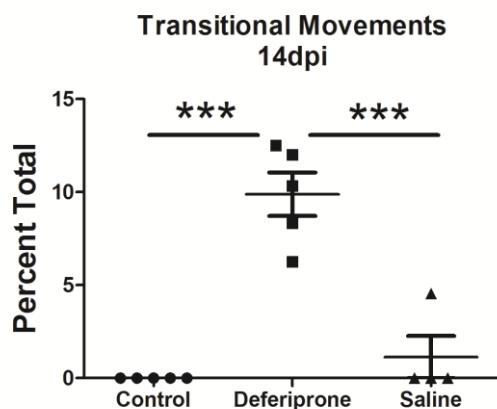
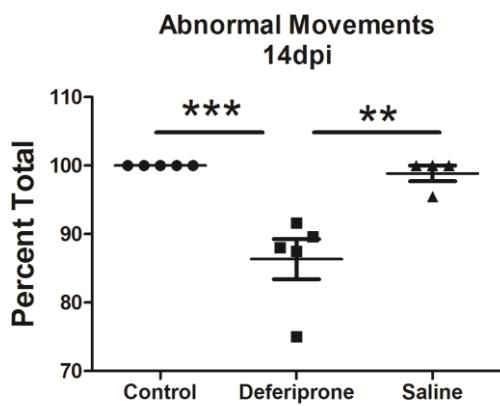
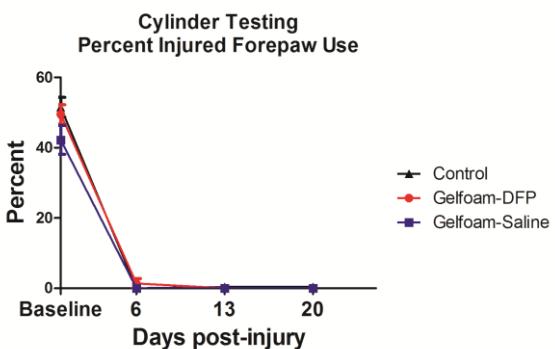
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Figure 17: Treatment with deferiprone improved forepaw function during the Pasta test but not the Cylinder test

Animals administered deferiprone showed no improvements in normal forepaw movements (A). However, deferiprone treated animals did show an increase in the percentage of transitional movements (B) and a decrease in abnormal movements (C). No differences were observed between any groups following the Cylinder test.

Following deferiprone treatment, injured spinal cord tissue was analyzed for changes in iron accumulation and the iron storage proteins H- and L-ferritin. No differences were observed in either the accumulation of iron or the iron storage proteins (Figure 18 A-C). Similarly, no differences were observed in the amount of astrocyte activation using the marker glial fibrillary acidic protein (Figure 18D). Even though there were no differences in the accumulation of iron in the spinal cord, treatment with deferiprone protected oligodendrocytes in the spinal cord white matter (Figure 19A) and dorsal funiculus (Figure 19B), but not in grey matter (Figure 19C). These data suggest the deferiprone treatment reduced the toxicity of the accumulated iron, even though it did not remove the iron from the spinal cord.

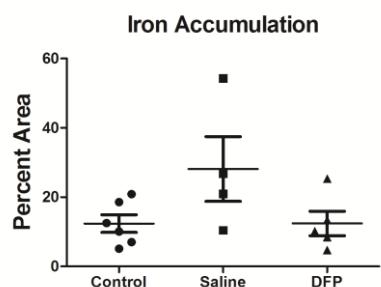
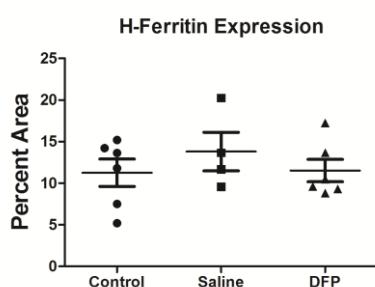
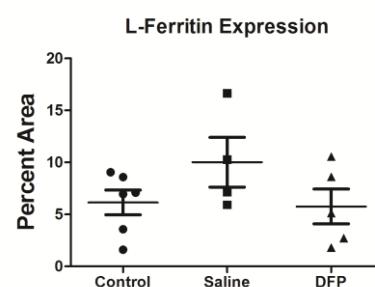
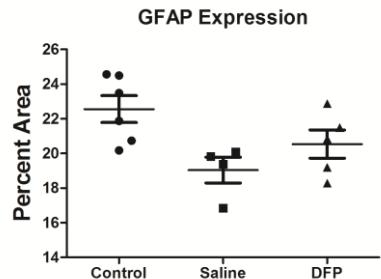
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Figure 18: Treatment with deferiprone did not alter iron accumulation, ferritin expression, or astrocyte activation.
Compared to controls, animals administered deferiprone had no differences in iron accumulation (A), H-ferritin expression (B), L-Ferritin expression (C), or astrocyte activation (D).

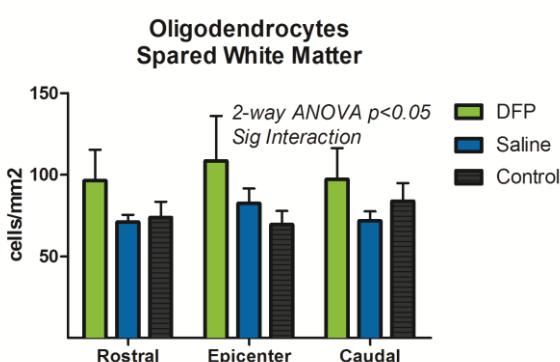
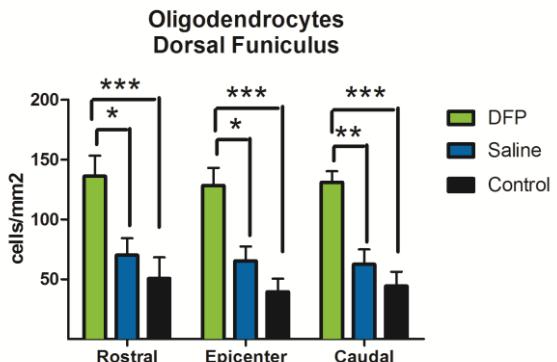
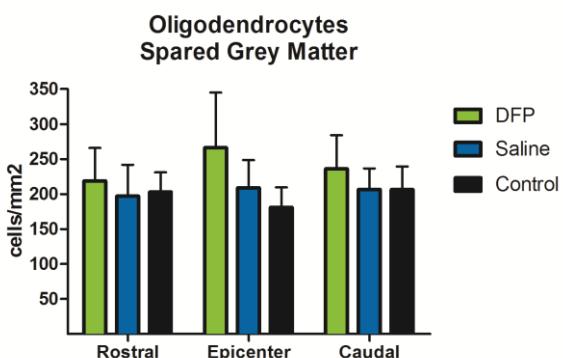
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Figure 19: Treatment with deferiprone spared oligodendrocytes in the white matter.

Animals administered deferiprone showed a significant main effect on oligodendrocyte counts in spared white matter (A) and a significant between group effect in the dorsal funiculus (B). No differences in oligodendrocyte counts were observed in the spared grey matter (C).

Key Research Accomplishments:

- Early treatment (1.5 h post-injury) with the iron chelator Exjade can result in improved locomotion following spinal cord injury.
- Delayed treatment (1 week post-injury) does not improve locomotion following spinal cord injury
- Treatment with Exjade at 80mg/kg/day does not change post-injury outcome
- Treatment with Exjade at 160mg/kg/day results in both locomotor and histological improvements after spinal cord injury with no significant side effects
- Treatment with Exjade at 320mg/kg does not result in improvements after spinal cord injury and had significant toxic side effects
- Early treatment with Exjade at 160mg/kg/day does not reduce oligodendrocytes after spinal cord injury
- Analysis of urine and liver iron content confirms oral Exjade successfully chelates systemic iron.
- Even though systemic iron levels were reduced, oral Exjade treatment does not reduce intra-spinal iron.
- Replication of previously effective dose, 160mg/kg/day for 1 week, failed to show improved locomotor function or tissue sparing.
- Analysis of chronic behavioral functions showed no difference in open field locomotion or hind limb sensitivity after Exjade treatment.
- The lack of robust or detectable effects with Exjade after contusive spinal cord injury provides evidence that oral Exjade is not a robust treatment for spinal cord injury.
- To improve the chance of seeing an effect after spinal cord hemisection, Exjade was replaced with a chelator with greater membrane permeability (the FDA approved iron chelator Deferiprone).
- Administration of deferiprone after a contusive spinal cord injury results in improved locomotor function, grey and white matter tissue sparing, and neuron sparing.
- Administration of deferiprone after cervical spinal cord hemisections results in improved forepaw function and improved oligodendrocyte sparing.

Reportable outcomes:

Publication of research:

2013 A.D. Sauerbeck , DL Schonber, JL LawJL, DM McTigue *Systemic iron chelation results in limited functional and histological recovery after traumatic spinal cord injury in rats*. Exp Neurol. 2013 May 24;248C:53-61

Presentations of research:

- A. D. SAUERBECK, A. T. LASH, D. M. MCTIGUE. Iron chelation following spinal cord injury attenuates functional and histological deficits. 2012 Military Health Services Research Symposium. Fort Lauderdale, Florida.
- A. D. SAUERBECK, D. L. SCHONBERG, A. T. LASH, D. M. MCTIGUE. Iron chelation following spinal cord injury attenuates functional and histological deficits. 2011 Society for Neuroscience Conference. Washington, DC.
- A. D. SAUERBECK, D. L. SCHONBERG, A. T. LASH, D. M. MCTIGUE. Iron chelation following spinal cord injury attenuates functional and histological deficits. 2011 Ohio State University Neuroscience Research Day

Funding received based upon findings from these studies:

2013 Neilsen Foundation Research Grant.

Title: **Novel iron targeting strategies to promote recovery from spinal cord injury**

Primary Investigator: **A.D Sauerbeck**

Funding applied for:

- Ohio State University, Center for Clinical and Translational Science Research Grant

- Title: Iron chelation following acute spinal cord injury will improve locomotor and histological outcomes.
- Primary Investigator: Dana McTigue, PhD
- NIH Postdoctoral Training Fellowship
 - Title: Alpha-synuclein is essential to spinal cord injury pathology
 - Primary Investigator: Andrew Sauerbeck, PhD
- The Morton Cure Paralysis Fund
 - Title: Alpha-synuclein is essential to spinal cord injury pathology
 - Primary Investigator: Andrew Sauerbeck, PhD
- The Craig H. Neilsen Fund
 - Title: Promoting recovery from spinal cord injury with novel iron chelator targeted strategies
 - Primary Investigator: Andrew Sauerbeck, PhD
- Paralyzed Veterans of America
 - Title: Novel iron targeting strategies to promote recovery from spinal cord injury
 - Primary Investigator Andrew Sauerbeck, PhD

Conclusions:

These studies indicate that iron chelation with the FDA approved drug Exjade can be therapeutically effective after spinal cord injury; however, the beneficial effects are not robust or consistent. The present studies suggest that a bell-shaped dose response curve for Exjade may exist where 160mg/kg/day is effective while 80 and 320mg/kg/day Exjade are not effective. These experiments show that iron chelation can result in both histological and locomotor improvements after spinal cord injury. Attempts to replicate the therapeutic benefits of Exjade were unsuccessful. To improve the ability to chelate intra-spinal iron, new experiments were conducted with deferiprone. Intrathecal administration of deferiprone beginning immediately after a contusive spinal cord injury results in locomotor improvements and tissue sparing. Administration of deferiprone directly to the site of a spinal cord hemisection with the use of gel foam promoted functional and histological changes. Animals that received deferiprone after the hemisection showed improvements in forepaw use with the Pasta test as well as increased oligodendrocyte sparing in the white matter. These results indicate that targeted direct administration of deferiprone to either a contusive or hemisection spinal cord injury promotes tissue sparing and recovery.

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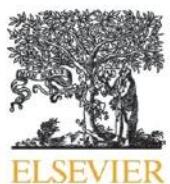
Appendix:

Title: Iron chelation following spinal cord injury attenuates functional and histological deficits.

Authors: A. D. SAUERBECK¹, D. L. SCHONBERG², A. T. LASH¹, D. M. MCTIGUE¹;

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Abstract: Following traumatic injury to the spinal cord iron accumulates at the site of the injury and is involved in the cascade of events which take place in the hours and days following the initial insult. Multiple reports looking at the post-injury accumulation of iron have shown the accumulated iron to be detrimental by facilitating oxidative damage through the Fenton reaction. Interestingly, in contrast to the damaging effects of iron, it has recently been shown that iron is also required for maximal oligodendrocyte replacement after injury to the spinal cord. Given both the detrimental and beneficial roles of iron following spinal cord injury, finding an effective method to therapeutically target the accumulated iron must serve to block the damaging mechanisms while also preserving the beneficial effects of iron. In the present studies, administration of the iron chelator Exjade was begun either one hour or seven days following a contusion spinal cord injury in rats in order to target either the early or delayed iron dependent events. When treatment was begun one hour following the injury, animals exhibited better locomotor function as well as more spared grey matter. Delaying the treatment until after the critical period of oligodendrocyte replacement did not result in any neuroprotective effects, suggesting that attenuating the damaging properties of iron which occur early after injury are of critical importance. Additionally, the early treatment with Exjade did not result in a reduction in the number of mature oligodendrocytes at one week following the injury which suggests the doses utilized in these experiments are capable of preserving locomotor function without impairing the post-injury replacement of oligodendrocytes. These studies show that following traumatic injury to the spinal cord treatment with the iron chelator Exjade is effective at attenuating deficits which occur after the injury and leads to improved locomotor function and tissue sparing.



Systemic iron chelation results in limited functional and histological recovery after traumatic spinal cord injury in rats



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Excess iron accumulation within the spinal cord is thought to exacerbate tissue damage and limit functional recovery after traumatic spinal cord injury (SCI). An optimal treatment to reverse or prevent damage would be to deliver an iron chelator systemically. Thus, we tested oral delivery of deferasirox (Exjade) in multiple studies using a rat model of mid-thoracic spinal contusion. Female Sprague-Dawley rats received a moderate contusion at vertebral level T8 and were given daily deferasirox for the first 7 or 14 days post-injury. The first two studies showed modest improvements in hindlimb function with limited improvement in tissue sparing. Two subsequent experiments to assess chronic functional changes and test longer-duration treatments failed to produce significant improvements. Testing a 2-fold higher deferasirox dose resulted in toxic side effects. To verify iron chelation treatment was effective, hepatic iron levels were measured which revealed that deferasirox robustly and significantly reduced systemic iron levels. Overall, this study suggests that oral iron chelation with deferasirox may lead to small but significant improvements in locomotor recovery or tissue sparing. However, given the lack of robust beneficial effects combined with potentially detrimental side effects such as exacerbated systemic anemia, oral administration of iron chelators may not be ideal for minimizing intraspinal iron-mediated pathology after SCI.

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Introduction

Every year in the United States there are ~ 11,000 new spinal cord injuries (SCI), which result in more than 250,000 people currently living with the chronic pathology associated with SCI. The initial insult leads to significant functional deficits, which have a devastating impact on the individual's health and quality of life. Even though substantial improvements in care have increased survival rates, people with SCI now live with significant deficits for many decades. The average lifetime cost for treating SCI can vary between \$1,400,000–\$4,300,000 depending on the severity and spinal level of the injury (NSCISC, 2011). Thus, given the financial obligations and the permanently reduced quality of life after SCI, finding effective therapeutic agents is critically needed.

Following SCI, damage occurs not only to nervous tissue but also to the surrounding vasculature, which results in intraspinal hemorrhage (Noble and Wrathall, 1985, 1989a, 1989b) accompanied by deficits in tissue perfusion (Mautes et al., 2000). The presence of intraparenchymal blood plays a significant role in secondary injury

processes further exacerbating tissue loss (Mautes et al., 2000). As hemoglobin from the extravasated blood is broken down, iron is released into the spinal cord leading to the production of damaging free radicals and propagation of secondary injury cascades (Liu et al., 2003, 2004; Zhang et al., 1996). For instance, iron is directly involved in the catalytic production of potent hydroxyl radicals through the Fenton reaction. Hydroxyl radicals can kill neurons (Bao and Liu, 2004) and also lead to progressive expansion of membrane damage by lipid peroxidation (Liu et al., 2004). Increased lipid peroxidation, evident by 3 h post-injury and persistent at the epicenter for at least 2 weeks (Carrico et al., 2009), demonstrates how a rapid rise in intraspinal iron can lead to long-lasting degradative cascades.

The role of iron in the progression of the secondary injury following SCI is further supported through work using iron chelators that can reduce tissue damage and promote functional improvement (Klapka et al., 2005; Paterniti et al., 2010; Rathore et al., 2008; Schultke et al., 2003, 2010a, 2010b). Although iron chelation therapy improved recovery, differences in study designs and drugs necessitate further investigation before studies should be initiated in humans. Previous work using the FDA approved iron chelator deferoxamine showed that targeting iron can attenuate post-SCI pathology (Paterniti et al., 2010). A caveat, however, is that the drug was given 30 min prior to injury. The iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) given intraperitoneally induced a delayed improvement in locomotor function in mice after SCI, suggesting that targeting chronically elevated

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intraspinal iron may be therapeutic (Rathore et al., 2008). In a different approach, a single injection of the iron chelator 2,2'-bipyridine (BPY) or BPY-5,5'-dicarboxylic acid directly into the spinal cord after spinal transection reduced the formation of the glial scar (Klapka et al., 2005; Weidner et al., 1999). However, these same studies show conflicting results as to whether treatment leads to axon regeneration and functional improvement (Klapka et al., 2005; Weidner et al., 1999). Unfortunately, SIH and BPY-DCA are not FDA approved and would require additional hurdles before being translated to human usage. Treatment with the flavonoid quercetin starting 1 h after compression SCI in rats had a variable effect on improving locomotor function in that a moderate dose improved stepping ability but higher doses had no effect (Schultke et al., 2003, 2010a, 2010b). Thus, given the diverse nature of the pre-clinical research on post-SCI iron chelation, determining the best candidate drug and treatment paradigm is essential before trials can be considered for humans. Further, our previous work showed that iron is essential for intraspinal oligodendrocyte genesis following spinal inflammation (Schonberg and McTigue, 2009; Schonberg et al., 2007). Therefore, it is equally important to ensure that iron chelation after SCI does not adversely affect anatomical or functional recovery by limiting the endogenous replacement of oligodendrocytes.

In the present studies, we tested the efficacy of the FDA approved iron chelator deferasirox after a clinically relevant spinal contusion injury. This drug is appealing because it can be given orally and is known to cross the blood-brain barrier. Therefore, we tested different administration protocols to determine if post-SCI treatment with deferasirox improved locomotor recovery and reduced tissue loss. Furthermore, given the necessity of iron for oligodendrocyte genesis, we also determined if oligodendrocyte numbers were altered by post-SCI iron chelation.

Methods

Injury and drug treatment

Spinal cord contusions were performed using standardized protocols as previously described (Almad et al., 2011; Almad and McTigue, 2010). All procedures conformed to NIH and The Ohio State University animal care guidelines. Briefly, adult female Sprague-Dawley rats (250 g; Harlan, Houston, TX) were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and a dorsal laminectomy was performed at the T8 vertebral level. Rats then received a moderate spinal contusion injury using the Infinite Horizons device (Precision Systems and Instrumentation) with a preset force of 200 kD (actual forces ranged from 200 to 219 kD with an average force of 205 kD). The muscles overlying the spinal cord were sutured and the skin was closed with surgical clips. Animals were given 5 ml of saline and placed into warm recovery cages immediately following the injury. Postsurgical care included 5 days of antibiotic treatment (gentamicin, 5 mg/kg) and saline to maintain hydration, and twice-a-day manual bladder expression until spontaneous voiding returned. Animals were randomly assigned to drug, vehicle, or control groups prior to beginning the treatments. Deferasirox (Exjade, Novartis, Basel, Switzerland) was obtained as 500 mg tablets and manually crushed into a fine powder prior to being dissolved in water at a concentration of 160 or 320 mg/kg in 0.5 ml water. Drug was delivered by oral gavage. Vehicle control animals received 0.5 ml of water by oral gavage. To control for any effect of gavage and extra handling, injury control animals receiving no gavage treatment were included in most studies. Administration of deferasirox or vehicle was begun at 1.5 h post-injury and continued once daily for the first week post-injury. In humans, deferasirox has a circulating half-life greater than 10 h for doses over 80 mg/kg (Galanello et al., 2003). Though an accurate half-life for orally administered deferasirox has not been well established in rats, oral administration of 100 mg/kg results in a plateau of peak serum levels for up to 8 h (Bruun et al., 2008), indicating that the half-life in rodents for doses over

100 mg/kg is greater than 8 h. Thus, rats were given one treatment per day. Over the course of the studies, some deaths were linked to treatment with deferasirox. In total, 6 of 52 rats died after treatments at 160 mg/kg/day, and 5 of 8 rats died after treatments at 320 mg/kg/day. No deaths occurred in control animals or those treated with vehicle.

Behavioral analysis

Animals were assessed for baseline locomotor function prior to surgery. After SCI, two reviewers blinded to study groups simultaneously evaluated each animal for 4 min using the BBB locomotor rating scale (Basso et al., 1995). Animals were assessed on 1, 3, 7 and 10 days post-injury (dpi) and then once a week thereafter. Exclusion criteria based on BBB scores were set prior to beginning the experiments to ensure only animals with similar injury severities were compared. Rats with BBB scores > 3 at 1 dpi or > 10 at 7 dpi were excluded for being too mild, and BBB scores ≤ 5 at 7 dpi were excluded as too severe. Across all of the studies, 2 of 14 controls, 3 of 32 vehicle, and 5 of 30 deferasirox animals were excluded.

Urinalysis

Iron content in the urine was assayed using the liquid ferrozine method (Iron Reagents Kit, Thermo Scientific). Urine was collected from awake animals and frozen at -80°C until analysis. Background absorbance was obtained at 560 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, California). A final absorbance reading was then taken at 560 nm in the presence of ferrozine. The concentration of iron was determined by subtracting the initial background absorbance for each sample from the final absorbance reading and then determining sample concentrations by comparing them with the standard curve based on known concentrations of ferrous iron.

Histological analysis

At the appropriate time post-injury, animals were given a lethal dose of ketamine (120 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.) and then transcardially perfused with 0.1 M phosphate buffered saline (PBS) until the tissue was cleared of blood. Next, animals were perfused with 400 ml of 4% paraformaldehyde (PFA). The spinal cord and liver were removed and post-fixed in 4% PFA for 2 h followed by phosphate buffer overnight. The next day, the tissue was transferred to 30% sucrose for 3 days prior to freezing and blocking for tissue sectioning. Tissue sections were cut at 10 μm using a cryostat and slide mounted (Superfrost Plus Slides, Fisher Scientific); slides were stored at -20°C until used. For tissue analysis, the following targets were visualized using immunohistochemistry: neurofilament (DSHB, RT97, 1:2000), neurons (Chemicon, NeuN, MAB377 1:50,000), macrophages (Serotec, OX42 MCA275, 1:2000), ferritin (Abcam, L-ferritin, ab69090 1:1000; H-ferritin ab65080 1:500), and oligodendrocytes (Abcam, CC1 ab16794, 1:800). Eriochrome Cyanine was used to visualize myelin for white matter sparing and the Perls Prussian Blue stain (Polysciences #24199-1) was used with DAB intensification to visualize iron.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 (San Diego, California). For behavioral and histological analysis a two-way repeated measures ANOVA was performed followed by post-hoc analysis to determine between group differences. For analysis of urine, liver, and spinal cord iron content, a one-way ANOVA was performed followed by post-hoc analysis to determine group differences.

Results

Iron accumulates at the injury site by 12 h post-injury and remains in macrophages chronically

By 12 h, hemorrhage is evident in lesion epicenter and peaks at 1 dpi. The intraparenchymal blood is a significant source for intraspinal iron (Fig. 1). High magnification of Perls stained tissue for ferric iron reveals that at 1 dpi, iron is contained within cells that resemble red blood cells (RBCs) detected in the hemorrhagic area (Fig. 1B). By 1 week post-injury, these cells are almost completely eliminated from the lesion center; however, dense iron accumulation remains in the lesion for at least 42 dpi (Figs. 1C, D). Macrophages within the injured tissue engulf RBCs and cellular debris, leading to increased intracellular iron levels and subsequent upregulation of ferritin, the major iron storage protein. The distribution of ferritin-positive macrophages corresponds to the localization of iron (Figs. 2A–C), suggesting that the persistent presence of iron after SCI is at least partially the result engulfment of red blood cells and then chronic iron storage by macrophages.

Systemic treatment with deferasirox reduces peripheral iron content

To verify that deferasirox effectively chelated iron in treated rats, a standard iron assay was used to measure iron content in urine collected prior to injury and at 1 dpi from vehicle and drug-treated rats. Vehicle- and deferasirox-treated rats both had elevated urine iron concentrations compared to pre-injury baseline levels (Fig. 3A), indicating that systemic iron loss is an acute feature after SCI. Deferasirox-treatment, however, resulted in a 4-fold increase in urinary iron compared to vehicle treatment at 1 dpi (Fig. 3A). Therefore, deferasirox treatment enhanced systemic iron removal acutely following SCI with urine iron content in treated rats returning to vehicle levels by 3 dpi. The return to baseline levels is most likely due to the main route of deferasirox excretion being through biliary elimination.

To evaluate systemic iron levels after the 7-day drug treatment, liver sections were collected from deferasirox, vehicle, and control rats to measure iron content, since the liver is a major iron storage organ.

Histological iron detection revealed a significant reduction in liver iron content in deferasirox-treated rats compared to both control groups (Figs. 3B, C). Indeed, iron was almost completely absent from the liver verifying that deferasirox potently removes systemic iron.

Acute treatment with deferasirox improves locomotor recovery after SCI

Our moderate contusion model of traumatic SCI is characterized by acute locomotor dysfunction with near complete paralysis at 1 dpi followed by progressive spontaneous recovery. In rats receiving daily deferasirox treatment (160 mg/kg/day) starting 1.5 h after SCI through 7 dpi, hindlimb function significantly improved at 4 and 7 dpi compared to non-treated rats (Fig. 4A). Interestingly, vehicle-treated rats also performed better than non-treated controls at 7 dpi (Fig. 4A). At 7 dpi, the majority (67%) of deferasirox-treated rats could perform plantar stepping compared to only 25% of vehicle-treated and 14% of control animals (Fig. 4B). Treatment with a lower dose of deferasirox (40 mg/kg/day or 80 mg/kg/day) did not alter locomotor or stepping recovery compared to controls (data not shown).

Iron chelator treatment for 7 dpi improved gray matter sparing and attenuated neuron loss

Treatment with deferasirox for 1 week post-injury (160 mg/kg/day) had no effect on white matter sparing but significantly improved gray matter sparing caudal to the injury epicenter (Figs. 5A–D). Similarly, cross-sections 1.8 mm caudal to epicenter contained significantly more NeuN positive neurons compared to controls (Fig. 5E). The lower deferasirox dose (80 mg/kg/day) had no effect on tissue sparing (data not show).

Deferasirox treatment did not alter macrophage accumulation, iron levels, or ferritin expression in the lesion

Since macrophages are active participants at the lesion site and are known to sequester and release iron, we determined if iron chelation altered macrophage accumulation. Following the 7-day deferasirox

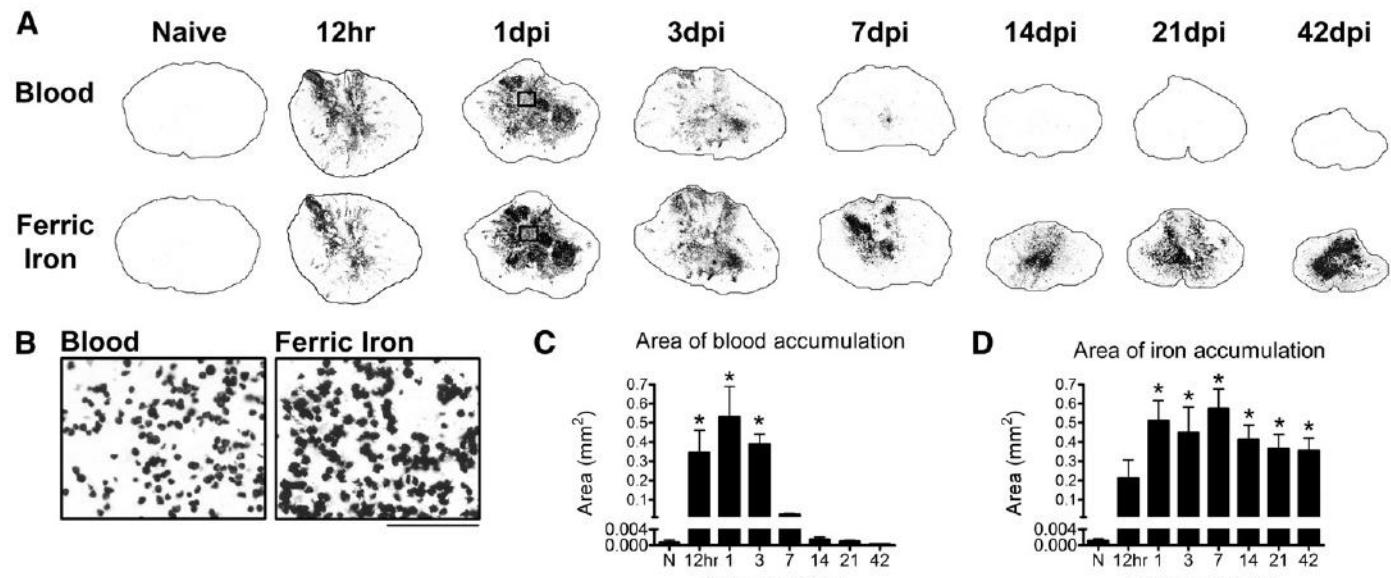


Fig. 1. Iron and red blood cells infiltrate the lesion by 12 h post-injury and iron remains elevated chronically. A) Blood from intraspinal hemorrhage is evident in the injury site by 12 h post-injury and is markedly reduced after 3 days post-injury (dpi). Adjacent sections labeled with the Perls stain to visualize ferric iron reveal a close juxtaposition of iron and blood components through 3 dpi. Thereafter, blood is minimal but dense intraspinal iron remains within the tissue. B) High magnification of boxes from 1 dpi sections in A shows that the localization of iron is morphologically similar to that of infiltrating cells. C) Quantification of the area of blood in cross-sections from each time point shows that blood is significantly elevated for 3 dpi in the injured spinal cord. D) Quantification of Perls reactivity shows significant elevation of intraspinal iron by 12 h post-injury that is maintained for at least 42 dpi. C: *p < 0.05 vs. Naive. D: *p < 0.05 vs. N

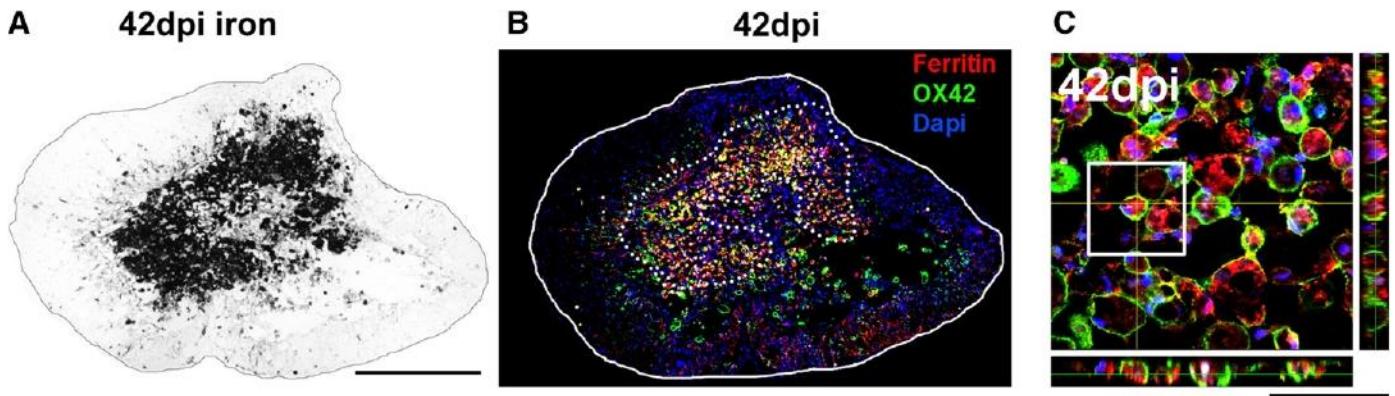


Fig. 2. Co-localization of iron and ferritin-positive macrophages after SCI. A) 42 dpi spinal cord section labeled with Perls stain to reveal distribution of iron. B) Confocal image of section adjacent to that in A immunolabeled for ferritin (red) and macrophages (green). The dotted lines outline the predominant localization of Perls stained iron from adjacent section. C) High power z-stack and orthogonal plane from center of lesion in B showing ferritin expression is mainly located within macrophages.

regimen, there were no observed changes in macrophage distribution, along with no discernible differences in iron accumulation as noted with Perls stain (Figs. 6A, B). However, a limitation of this iron detection method is that it does not distinguish between chelated and non- chelated iron. A required step of the Perls stain is acid treatment, which induces the release of deferasirox-bound iron thereby enabling its detection. To circumvent this technical issue, we used ferritin expression as a surrogate to measure intracellular iron levels. Expression of L-Ferritin, a subtype predominantly located in macrophages, was not altered by deferasirox treatment (Fig. 6C); however, there was a trend for reduced H-Ferritin, a subtype mainly present in oligodendrocytes and macrophages (Fig. 6D, $p = 0.077$). Additionally, deferasirox treatment did not alter Collagen IV expression (data not shown), which was shown previously to be reduced following direct administration of an iron chelator to the injured spinal cord (Klapka et al., 2005; Weidner et al., 1999).

Deferasirox treatment does not change oligodendrocyte numbers post-injury

Previous work by our group showed that marked oligodendrocyte genesis occurs along post-SCI lesion borders (Tripathi and McTigue, 2007), and that iron is essential for maximal oligodendrocyte

replacement following intraspinal macrophage activation (Schonberg and McTigue, 2009). Therefore, we quantified oligodendrocytes in spared tissue and lesion borders to determine if iron chelation negatively (or positively) affected oligodendrocyte numbers. Post-SCI treatment of deferasirox did not change oligodendrocyte numbers in spared white matter or lesion borders (Fig. 7).

Acute deferasirox treatment after SCI does not result in a long-term recovery benefit

An additional cohort of animals was used to not only replicate our previous acute functional results (Figs. 4 and 5) but also determine if locomotor scores continue to improve beyond 7 dpi. As expected, deferasirox treatment (160 mg/kg/day for 7 dpi) improved locomotor recovery at 4–7 days post-injury and resulted in similar plantar stepping performance as in our first study (Figs. 8A, B). However, by 10 dpi, there was no statistical difference in BBB scores between all groups. Contrary to our earlier results, deferasirox treatment did not result in improved gray or white matter tissue sparing (Figs. 8C, D) or neuroprotection (data not shown). Similar to the first study, treatment with deferasirox did not alter intra-spinal iron accumulation,

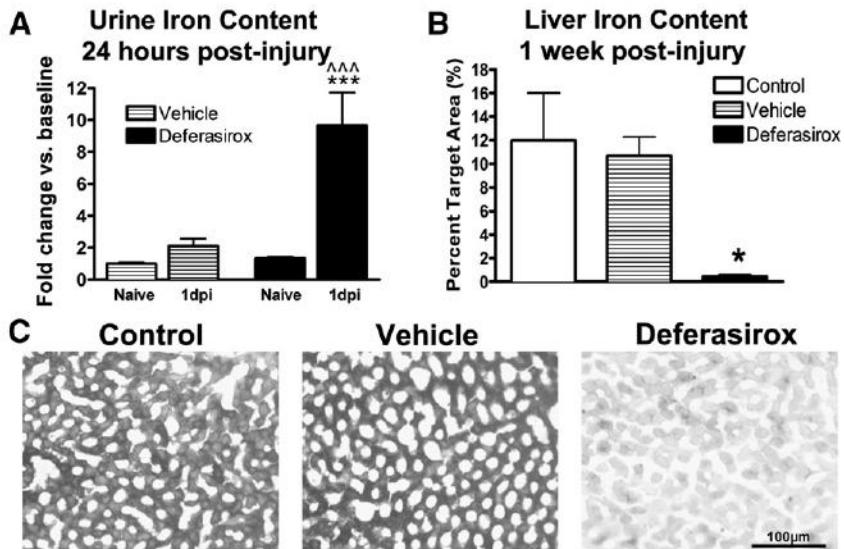


Fig. 3. Systemic treatment with deferasirox reduces peripheral iron content. A) Urinary iron content was doubled at 1 dpi in control animals compared to pre-injury levels. Urine from rats receiving deferasirox had significantly more iron compared to pre-injury values and to 1 dpi vehicle-treated values. B) One week after daily deferasirox treatments (160 mg/kg/day), liver iron content was significantly decreased. C) Representative images of liver sections stained for iron in black showing a significant reduction in hepatic iron following deferasirox treatment. A: ***p < 0.001 deferasirox 1 dpi vs. vehicle 1 dpi; ^p < 0.01 deferasirox 1 dpi vs. naive baseline. B: *p < 0.05 deferasirox vs. control and vehicle.

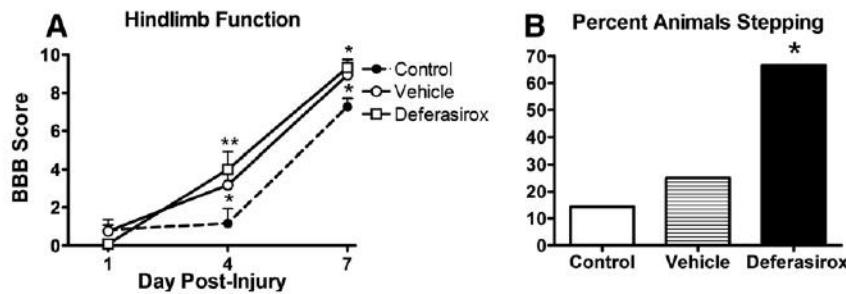


Fig. 4. Acute treatment with deferasirox attenuates locomotor deficits after SCI. A) Daily deferasirox treatment significantly improved locomotor function compared to control animals. Animals given the vehicle (water without deferasirox) also exhibited improved locomotor function compared to controls. B) After treating rats with deferasirox for 1 week, significantly more animals could plantar step at 7 dpi (66%) compared to control (14%) and vehicle (25%) groups (Chi square test). A: 4 dpi *p < 0.05 vehicle vs control **p < 0.01 deferasirox vs control, 7 dpi *p < 0.05 control vs vehicle and deferasirox; B: *p < 0.05 deferasirox vs. control and vehicle.

macrophage infiltration, or the number of mature oligodendrocytes (data not shown).

Replication of early deferasirox treatment or increasing the dose of deferasirox did not replicate functional improvements after SCI (Studies 3 and 4)

In Study 3, a cohort of animals received vehicle or deferasirox and survived for 9 weeks post-injury to assess chronic changes. Contrary

to Studies 1 and 2, treatment for 7 dpi with deferasirox did not improve acute BBB scores (Fig. 9A). Since this was a chronic study, we assessed changes in hindlimb sensitivity; no differences in sensitivity or development of allodynia were detected (data not shown). Deferasirox treatment also did not alter gray or white matter sparing measured at 9 weeks post-injury (Figs. 9B, C).

In Study 4, we extended deferasirox treatment for 2 weeks post-injury, hypothesizing that longer iron chelation therapy would lead to sustained recovery. However, neither 7 day nor 14 day

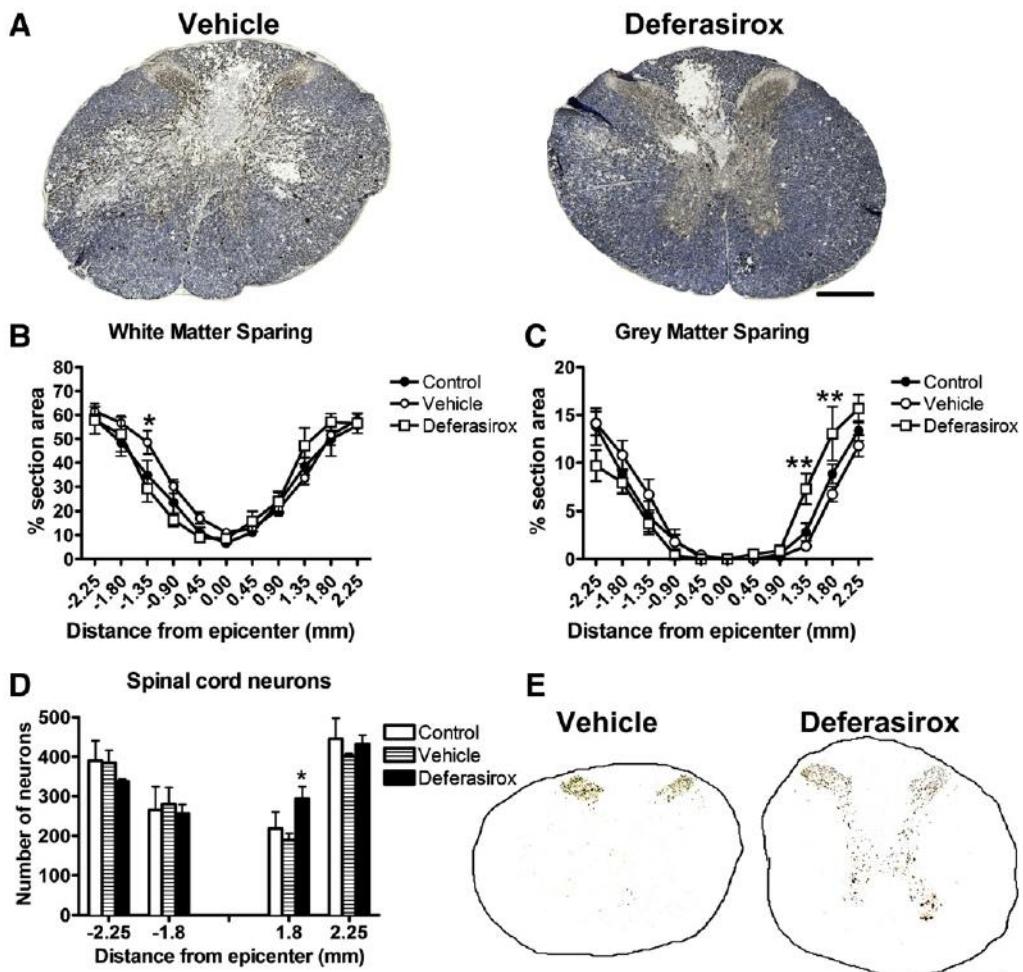


Fig. 5. Daily deferasirox for 7 dpi (160 mg/kg/day) attenuates tissue loss and neuron death following traumatic spinal cord injury. A) Representative images of sections 1.8 mm caudal to epicenter stained for myelin (blue) and neurofilament (black/brown). B) Stereological quantification of white matter sparing shows more white matter rostral to epicenter in vehicle-treated rats and a trend towards increased white matter sparing caudal to the epicenter in the deferasirox-treated group. C) Stereological quantification of gray matter sparing shows a significant increase in gray matter caudal to epicenter in deferasirox-treated animals. D) Quantification of neurons revealed deferasirox treatment increased neuron sparing at 1.8 mm caudal to epicenter. E) Representative images of sections 1.8 mm caudal to epicenter immunolabeled from NeuN from vehicle- and deferasirox-treated rats. B: *p < 0.05 vehicle vs. deferasirox; C: **p < 0.01 vehicle vs. deferasirox.

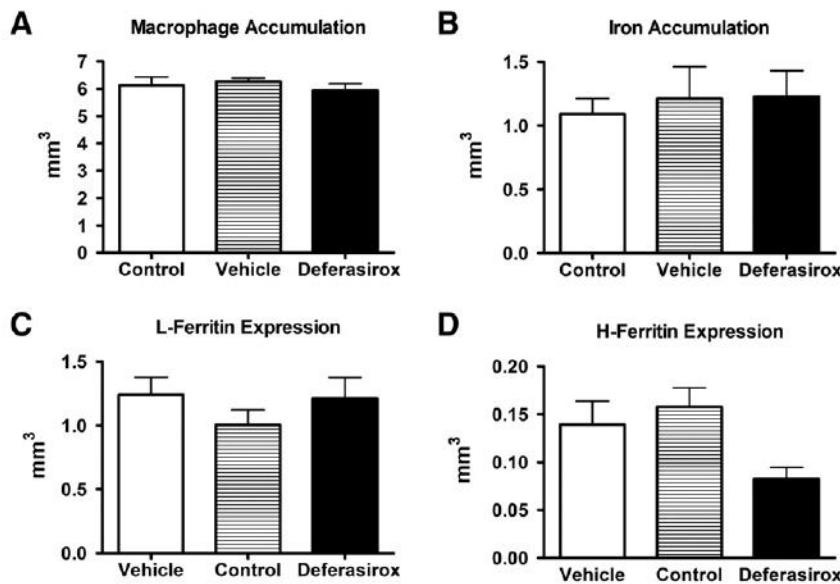


Fig. 6. Deferasirox treatment did not affect macrophage accumulation, intraspinal iron level, or ferritin expression in the lesion site. Treatment with deferasirox (160 mg/kg/day) during the first 7 dpi did not significantly alter A) macrophage accumulation B) iron accumulation C) expression of the iron storage protein L-Ferritin or D) expression of the iron storage protein H-Ferritin.

deferasirox treatment improved locomotor recovery in this study (Fig. 10). Lastly, we tested the efficacy of giving a higher dose of deferasirox (320 mg/kg/day) during the first week post-injury. This dosing regimen caused significant morbidity, which necessitated early termination of treatment. In these studies, reduced hepatic iron was again noted after treatment indicating that deferasirox was absorbed and chelated systemic iron.

Discussion

After traumatic spinal cord injury, our data and that of other studies clearly show a rapid and marked increase in intraspinal iron. This is accompanied by increased iron regulatory proteins such as ferritin and long-term storage in macrophages within and around the lesion sites. This situation sets the stage for acute and long-term iron-mediated damage. Acutely, as blood floods the spinal tissue, iron is released during red blood cell and hemoglobin degradation, and from carrier molecules such as circulating transferrin and ferritin. Release of free iron from carrier molecules will be facilitated by the acidic nature of the early post-injury environment (Munoz et al., 2011; Qi et al., 1995). While macrophages likely play an early protective role by taking up and storing iron (Olakanmi et al., 1993), chronically iron-loaded macrophages may function as a constant iron source that facilitates prolonged oxidative damage thereby limiting long-term repair and recovery. Indeed, studies have shown that iron-laden macrophages actively release iron and ferritin extracellularly over time (Yuan et al., 2004).

Given the highly reactive nature and known toxicity of iron, treatment with an iron chelator seems an obvious and promising strategy to protect spinal tissue after trauma and promote long-term recovery.

An ideal iron chelator would be deliverable systemically, have a long half-life and be able to chelate intracellular iron. Unlike many of the first generation iron chelators, FDA-approved deferasirox (Exjade) meets these criteria. According to company information, deferasirox enters the CNS after oral administration and studies have shown its half-life is > 10 h (Galanello et al., 2003). In addition, vascular leakiness and increased permeability would facilitate delivery of the chelator to the injury site. Since other iron chelators have shown promise as potential SCI therapies, we chose to test whether oral delivery of deferasirox would improve recovery after SCI in rats. Because intraspinal bleeding begins almost immediately upon injury, we delivered deferasirox at the earliest time point that rats could safely be gavaged after anesthesia (1.5 h post-injury). It is recognized that this would be difficult to achieve clinically, and would have been followed up with later delivery start times had beneficial effects been observed consistently.

We performed four experimental replicates. In Studies 1 and 2, improved locomotor function was noted during the drug treatment (1.5 h-7 dpi), which was evident by improved BBB scores and increased ability to perform weight supported plantar stepping at 7 dpi. In the first study, BBB scores for deferasirox treated animals were not statistically different from vehicle animals; however, the lack of a statistically significant difference does not mean there was

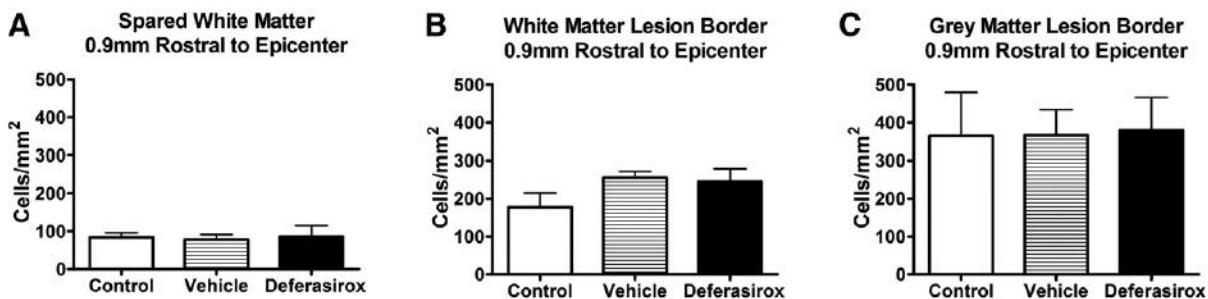


Fig. 7. Deferasirox treatment did not affect oligodendrocyte numbers post-injury. Administration of deferasirox (160 mg/kg/day) during the first week post-injury does not change

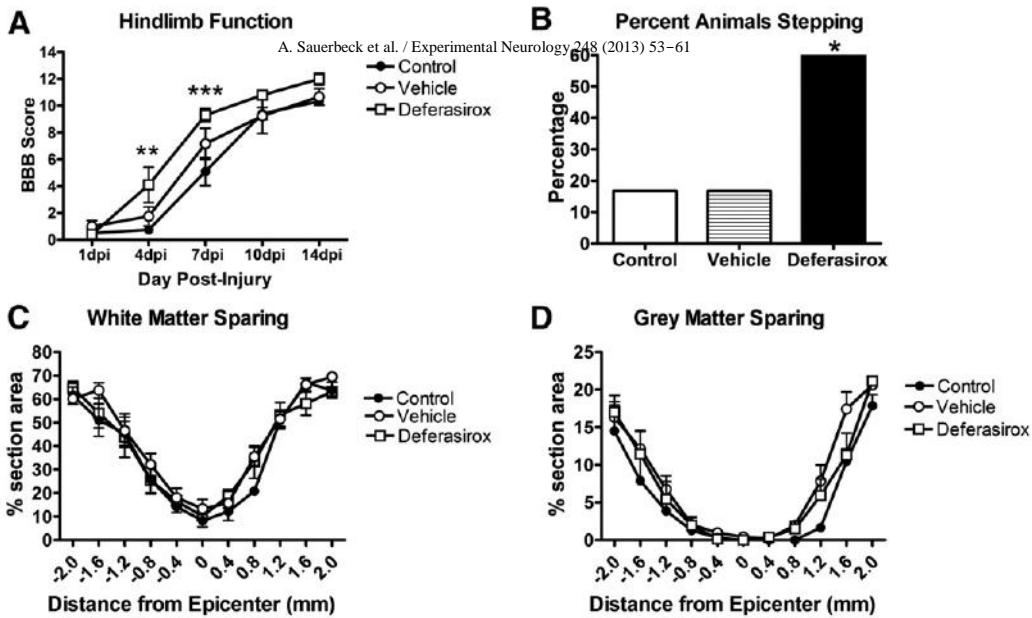


Fig. 8. Treatment with deferasirox for 1 week replicates acute functional recovery after SCI. A) Analysis of hindlimb locomotion using the BBB scale shows that deferasirox-treated rats had significantly improved locomotor function compared to control animals at 4 days and 7 dpi. B) More deferasirox-treated rats could perform weight supported plantar stepping than control or vehicle animals at 7 dpi. C, D) Stereological analysis of white and gray matter sparing showed no differences between any groups. A: **p < 0.01, ***p < 0.001 deferasirox vs. control.

not a functional difference. Given the ordinal nature of the BBB scale, it is important to consider what each score represents functionally. At 7 dpi, only 25% of vehicle treated animals could plantar step in contrast to 67% of deferasirox treated animals that could plantar step.

Even though the BBB data did not reach statistical significance, it is nonetheless a functionally significant improvement in locomotor function. In one of these studies, gray matter sparing and neuroprotection were significantly increased caudal to the injury site. However, since improved locomotor improvements were not sustained past the end of drug treatment, it is unlikely the functional recovery was due to improved tissue sparing. Further, given that these were mid-thoracic injuries, it is unlikely that improved neuronal sparing at this level would mediate improved walking ability. Instead, it may be due to a general improvement in the "health" of the spinal parenchyma at and around the injury site due to a decrease in iron-mediated dysfunction. At the time of drug treatment termination, intraspinal iron levels were still clearly elevated, especially within macrophages, indicating that deferasirox was unable to physically remove iron from the injured spinal cord.

The latter two studies unfortunately showed no benefit of oral iron chelator treatment on locomotor recovery or tissue sparing. Since fresh drug was prepared for each treatment, it is unlikely that the irreproducible functional and anatomical results could be

explained by reduced drug efficacy. Indeed, similar to other studies using this drug (Finkenstedt et al., 2010), 7 dpi liver histology confirmed that deferasirox potently chelated systemic iron stores. A possible explanation for differences between outcomes could be subtle inter-study variations in injury severity. Although the force and displacement biomechanics were similar between studies, there was a significant difference in mean forces between Study 2 (203 ± 2.58) and Study 4 (208 ± 5.15). Similarly, the average 7 dpi BBB scores for the deferasirox groups in Studies 3 and 4 were lower than those in Studies 1 and 2 (7.1, 8.0 vs. 9.3, 9.3). The vehicle treated groups showed a similar trend in 7 dpi BBB scores in Studies 3 and 4 compared to Studies 1 and 2 (7.6, 6.6 vs. 8.9, 7.2). One could speculate that the efficacy of chelation treatment may be dependent on the severity of the injury, which, if true, would mean translating our results to the clinic would be challenging given the wide variation of human SCI cases.

While deferasirox efficiently reduced systemic iron stores, we did not detect a measurable reduction intra-spinal iron after SCI. This may be due to the pharmacodynamics of deferasirox. The predominant mechanism whereby oral deferasirox removes iron from the body is by binding and eliminating iron systemically (Waldmeier et al., 2010). Circulating iron levels are easier to chelate than intra-cellular iron since they do not require deferasirox to passively cross a cell

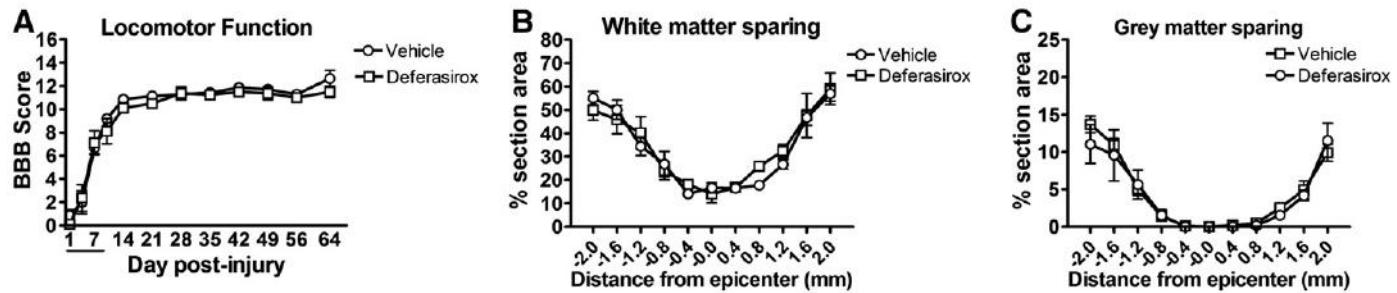


Fig. 9. Replication of early deferasirox treatment fails to reproduce functional improvements after SCI. A) Analysis of open field locomotion using the BBB scale showed no differences in hindlimb function up to 9 weeks post-injury. B, C) Giving deferasirox during the first week-post-injury did not alter gray or white matter tissue sparing. oligodendrocyte numbers in the A) spared white matter B) white matter lesion borders or C) gray matter lesion borders.

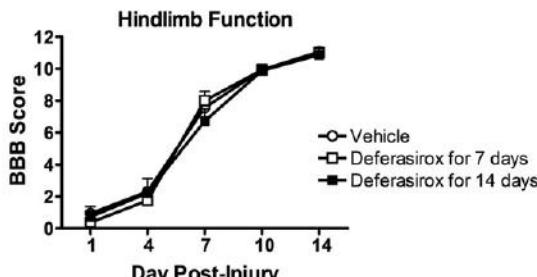


Fig. 10. Replication of 7 dpi deferasirox treatment or 14 dpi treatment fails to reproduce functional improvements after SCI. Animals were given deferasirox at 160 mg/kg/day for either 7 or 14 days post-injury and locomotor function was tested using the BBB scale. Neither treatment paradigm altered recovery of hindlimb function.

membrane. Once deferasirox binds iron, it forms an ~800 kD deferasirox-iron complex which does not exit cells (Hider and Zhou, 2005).

However, this intracellular chelation may still be beneficial by preventing free iron from participating in free radical production and other damaging cascades (Yu et al., 2009). Since methods such as the Perls prussian blue stain or atomic flame absorption spectrometry do not distinguish between free and deferasirox-bound iron, it is difficult to determine the amount of intra-spinal iron bound to deferasirox. However, even without definitively demonstrating that deferasirox chelated intra-spinal iron, our histological results indicate intra-spinal neuroprotection occurred (at least in one study) as a result of iron chelation.

After SCI, the proliferation of NG2 progenitor cells leads to the production of new oligodendrocytes within the spinal cord (McTigue et al., 2001; Tripathi and McTigue, 2007). These myelinating cells are an essential component of spinal cord physiology, proper CNS function, and post-SCI recovery. Furthermore, iron is required for new oligodendrocyte genesis, which can be reduced by deferasirox treatment (Schonberg and McTigue, 2009). However, in contrast to

reduced oligodendrocyte numbers in the TLR4-mediated inflammation model (Schonberg and McTigue, 2009), deferasirox administration following contusive SCI did not reduce the number of oligodendrocytes in the spinal cord. These differences may be attributable to a number of microenvironmental differences between the two models such as iron source and availability, microglial/ macrophage activation states, as well as mitogenic and stress signals for oligodendrocytes and their progenitors. Both models demonstrate that deferasirox can affect cellular phenotypes in the spinal cord without noticeable changes in absolute iron levels, suggesting that deferasirox reaches the spinal cord but is unable to efficiently eliminate iron from the lesion site. In CNS pathologies such as Parkinson's disease, where iron levels are elevated yet tissue is not damaged to the extent as after spinal contusion, peripherally administered deferasirox attenuates iron-mediated pathology by reducing cell death (Dexter et al., 2011). Taken together, these results suggest that deferasirox can produce neuroprotection; however, utilizing

CNS iron content as a measure of drug efficacy may not be reliable for deferasirox. Also, the excess damage induced by contusive injury may overwhelm the beneficial effects of modest iron chelation.

Overall our work and that of others provide tantalizing evidence that if the correct parameters can be defined, iron chelation may indeed be an effective strategy for improving tissue sparing and functional recovery after SCI. However, the ability to find the correct treatment parameters may be difficult or impossible with certain injury severities. In our study, excess iron is present in the spinal cord as early as 12 h post-injury, which is consistent with other injury models (Simard et al., 2007). Unpublished data from our lab show that less severe spinal contusion injuries exhibit a more delayed accumulation of intraspinal iron, meaning these may be more amenable to early chelator treatments. Furthermore, the marked systemic iron depletion detected in our study indicates that oral/systemic treatment

may not be the ideal approach, especially since anemia is often detected in SCI patients (Frisbie, 2010; Huang et al., 1990). Ongoing studies in our laboratory are testing CNS-targeted approaches to determine if local administration of a lipid soluble iron chelator is neuroprotective. This should avoid systemic side effects and hopefully allow physical removal of iron from the spinal cord, and, in turn, provide important insight into the role of intraspinal iron in post-injury cell death and dysfunction.

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